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(54) Title: PROCESS FOR THE PRODUCTION OF A POLYPEPTIDE

(57) Abstract

A process for the production of a polypeptide in which host cells transformed with DNA coding for a fusion protein comprising the polypeptide and a further peptide comprising a C-terminal secretion sequence are cultured to express and secrete the fusion protein. The C-terminal secretion sequence is, for example, the haemolysin C-terminal secretion sequence.

PROCESS FOR THE PRODUCTION OF A POLYPEPTIDE

Field of the Invention

5   This invention relates to recombinant DNA biotechnology and in particular to processes for the production of polypeptide products in transformed host cells wherein the polypeptide products are secreted from the host cells.

Background to the Invention

In recent years advances in recombinant DNA biotechnology have made it possible to produce a wide variety of useful polypeptide
10   products in host cells which have been transformed and transfected with DNA sequences which code for production of the polypeptide products.   Thus hormones such as insulin and growth hormones e.g. human growth hormone, and industrially or therapeutically useful enzymes, such as chymosin and tissue plasminogen activator (tPA) have
15   been produced in transformed host cells.

Bacterial cells, in particular E. coli, have been used as host cells for the production of recombinant polypeptide products.   The genetic systems of such bacterial cells are now relatively well understood and also such cells exhibit good growth characteristics.
20   However, when such bacterial cells are used to overproduce foreign proteins, the foreign products typically accumulate within the host cells and it is usually necessary to disrupt the cells to effect recovery of the products.   Also recombinant products are often produced within bacterial host cells in the form of insoluble
25   aggregates in which the polypeptides are not in their native, biologically functional form.   It is necessary, therefore, to solubilise and denature/renature the insoluble polypeptide products to obtain useful products in soluble, native, biologically functional form.   For instance, British Patent No. GB 2100732B describes inter
30   alia processes for production of methionine-prochymosin in E. coli involving disruption of the bacterial host cells and treatment with

urea or guanidine HCl to solubilise the unnatural prochymosin-
containing aggregates which are produced.   The processes of cell
disruption and denaturation/renaturation add significantly to the
cost of producing recombinant polypeptide products.

5   Attempts have been made therefore to develop bacterial
expression systems which secrete recombinant products into the
extracellular culture medium.   For example, recombinant heterologous
polypeptides have been expressed in bacteria as fusion proteins in
which the heterologous polypeptide sequence is joined with an
10   N-terminal signal sequence.   However, such fusion proteins, although
exported across the inner membrane in Gram-negative bacteria with
concomitant removal of the signal sequence, fail to cross the outer
membrane and remain within the periplasm.   Thus, it is still
necessary to disrupt the host cells to effect recovery of
15   heterologous recombinant products and denaturation/renaturation
treatment may be required to yield products in native, biologically
functional form.

          Also 'leaky' mutants of Gram-negative bacterial host cells such
as E. coli have been proposed for use in the production and secretion
20   of products to the extracellular medium.   However, such mutant cells
are often not suitable for large scale production of heterologous
protein products since the yield of product is generally low and the
fragility of the cells makes them unsuitable for growing on a large
scale.

25   Haemolysin (Hly) is an extracellular protein toxin which is
produced by some strains of E. coli, and as such is one of the few
proteins produced by Gram-negative bacteria which are transported
across both the cytoplasmic and outer membranes.   Hitherto, however,
the processes by which haemolysin is secreted to the extracellular
30   medium have not been satisfactorily explained.   Studies have
indicated that a specific transport system determined by two of the
hly genes is responsible for transport of haemolysin across the outer
membrane (Wagner et al, J. Bacteriol. 154, 200, 1983), and at least
four genes the hly A, hly B, hly C and hly D genes, are required to
35   elicit a cell-free haemolytic phenotype.   The hly C gene product

appears to be required for activation of the hly A gene product which provides the haemolytically active species; whereas the hly B and hly D gene products (previously referred to as hly Ba and hly Bb) are essential for transport of haemolytic activity to the extracellular

5 medium.

The primary product of the hly A gene does not contain an N-terminal signal sequence (Felmlee et al (1985) J. Bacteriol 163 p88 - 93). Thus, in an attempt to explain how secretion of the hly A gene product is achieved, Goebel and co- workers (Hartlein, M. et al,

10 J. Cell Biochem. 22, 87 - 97, (1983)) have produced a model, in which the product of the hly A gene (107 Kd) is activated by the hly C gene product itself and is processed, possibly by an autoproteolytic activity located near the C-terminal end of the Hly A protein, to yield a smaller (58 Kd) haemolytically active peptide which is

15 transported through the cytoplasmic membrane to the periplasm. They further propose that transfer of the 58 Kd haemolytically active peptide to the outer membrane and its release therefrom to the extracellular medium is promoted by the products of the hly B gene (46 Kd) and the hly D gene (62 Kd) respectively. Hartlein et al

20 have also proposed the generation (by a proteolytic cut) of an N-terminal end of the 58 Kd fragment which can act as a signal peptide permitting transport of the fragment across the cytoplasmic membrane in the usual manner.

However, this explanation of the mechanism of secretion of the

25 hly A gene product is controversial. Recently work in our laboratory and elsewhere has demonstrated the presence of large haemolytically active peptides, corresponding to the size of the initial hly A gene product, (107 Kd) in supernatants from cultures of haemolytic E. coli. This work suggests that cleavage of the Hly A

30 protein is not required for production of the active extracellular haemolysin.

Hitherto, however, a satisfactory explanation of the mechanism by which haemolysin is secreted into the extracellular medium has not been forthcoming.

We have further studied the haemolysin secretion system and have
shown that secretion of haemolysin from E. coli can be blocked by
deletion of 27 amino acids from the C-terminus of the Hly A protein
although this truncated molecule may be haemolytically activated by
5   the hly C gene product.   Further we have shown that a 23 Kd peptide
from the C-terminus of Hly A contains all the information necessary

for its own secretion.   We have concluded, therefore, that all the
information necessary for recognition and export of haemolysin in the
presence of the hly B and hly D gene products, which appear to locate
10   in the cell envelope, is contained within the 23 kilodalton
C-terminal fragment of the hly A gene product.   Our work constitutes
the first finding of a C-terminal secretion sequence.   Such
C-terminal secretion sequences may be used in the preparation of
recombinant fusion proteins which may be expressed and secreted from
15   host cells.

The Hly B protein shows extensive homology (170 identical or
conserved amino acid substitutions out of a stretch of 228 amino
acids in the C-terminal region (Gerlach et al Nature 324 485-489
(1986)) with the p-glycoprotein Mdr (Multi-drug resistant protein)
20   found in the surface of many drug resistant mammalian tumour cells.
The Mdr protein is directly responsible for drug resistance and
appears to function as an export pump (Ames, Cell 47 323-324
(1986)).   The p-glycoprotein and haemolysin systems may therefore
belong to a family of novel surface export mechanisms distinct from
25   other export processes.

Summary of the Invention

Accordingly, in a first aspect the present invention provides a
process for the production of a polypeptide, in which host cells
transformed with DNA coding for a fusion protein comprising the
30   polypeptide and a further peptide comprising a C-terminal secretion
sequence are cultured to express and secrete the fusion protein
therefrom.

In the context of the present description 'a C-terminal secretion sequence' denotes a sequence of amino acids present in the C-terminus of a secreted polypeptide which sequence comprises essential information required for recognition and secretion of the secreted polypeptide via its secretion pathway. Preferably the C-terminal secretion sequence is a haemolysin C-terminal secretion sequence.

Thus in a preferred embodiment of the first aspect the invention provides a process for the production of a polypeptide in which host cells transformed with DNA coding for a fusion protein comprising the polypeptide and a further peptide comprising a haemolysin C-terminal secretion sequence are cultured to express and secrete the fusion protein therefrom.

The phrase 'haemolysin C-terminal secretion sequence' denotes a sequence of amino acids containing essential information required for recognition and export via the haemolysin secretion pathway. This sequence characteristically comprises at least part of a C-terminal sequence of an authentic E. coli haemolysin toxin or a substituted or modified equivalent thereof, provided it functions as a haemolysin C-terminal secretion sequence. Preferably the secretion sequence comprises a sequence identical to at least part of an authentic E. coli hly A gene product C-terminal sequence. The E. coli hly A gene product C-terminus may contain one or more sequences which function as C-terminal secretion sequences. For example, a secretion sequence may be identical to one or more subsequences of the 218 amino acid C-terminal sequence of Hly A as shown in Figure 3. Without prejudice, we believe that at least one haemolysin C-terminal secretion sequence lies within the final 218 amino acid residues, especially within the final 150 amino acid residue, more especially within the final 113 amino acid residues and most especially within the final 32 amino acid residues of the Hly A C-terminus.

It will be appreciated, however, that provided the further peptide of the fusion protein contains a haemolysin C-terminal secretion sequence, it will function to promote secretion of the fusion protein. Therefore, the further peptide may contain further amino acids in addition to the secretion sequence. For example,

additional amino acids may be required to separate the secretion
sequence from the polypeptide in order advantageously to provide a
favoured steric configuration in the fusion protein which promotes
the secretion process.

Without prejudice to the generality of the present description,
we believe that it may be advantageous to separate the secretion
sequence from the polypeptide by up to 60 amino acids.

The process of the invention may be used for production of
polypeptides in general, including natural and synthetic polypeptides
and including polypeptides of bacterial origin.   Preferably,
however, the process may be used for production of heterologous
polypeptides, i.e. polypeptides which are foreign to the host cells,
especially eucaryotic polypeptides.   For example, the polypeptides
may be useful or therapeutic eucaryotic polypeptides such as
hormones, enzymes and interleukins.

In a second aspect the invention provides a fusion protein
comprising a first polypeptide and a further peptide in which the
further peptide comprises a C-terminal secretion sequence, preferably
a haemolysin C-terminal secretion sequence.

The fusion protein may comprise an internal fusion protein or an
N-terminal fusion protein i.e. a fusion protein in which the further
peptide containing the haemolysin C-terminal secretion sequence is
joined to the N-terminal of the polypeptide.   Preferably, however,
the fusion protein is a C-terminal fusion protein, e.g. a fusion
protein in which the further peptide containing the haemolysin
C-terminal secretion sequence is joined to the C-terminus of the
polypeptide.   Typically, the fusion proteins comprise selective
cleavage sites at the junction or junctions between the polypeptide
amino acid sequence and the amino acid sequence of the further
peptide.   Such selective cleavage sites may comprise one or more
amino acid residues which provide a site susceptible to selective
enzymatic, chemical or other cleavage.   The fusion protein may be
further processed to cleave the polypeptide therefrom; for instance,
if the polypeptide is required without additional amino acid

residues.   Thus, the process of the first aspect of the invention
includes a process in which, following secretion, the fusion protein
is cleaved to yield the polypeptide.

In a third aspect the invention provides a DNA sequence coding
for a fusion protein according to the second aspect of the invention.

The DNA sequence of the third aspect of the invention comprises
a first DNA sequence coding for the polypeptide and a second DNA
sequence coding for the further peptide and may include one or more
additional DNA sequences which code for one or more selective
cleavage sites at the junction or junctions between the first and
second DNA sequences.   The methods by which such DNA sequences may
be obtained and linked to provide the DNA sequence of the third
aspect of the invention are well known in the field of recombinant
DNA technology.   Thus DNA coding for the polypeptide may be obtained
from parent cells which produce the polypeptide;   for instance in the
form of a cDNA sequence prepared by reverse transcription of mRNA
obtained from the parent cells.   Also DNA coding for the further
peptide, containing a haemolysin C-terminal secretion sequence, may
be obtained from the 3' terminal region of an hly A gene by
appropriate restriction enzyme digestion.

Without prejudice to the generality of the present description,
we believe that at least one haemolysin C-terminal secretion sequence
may be present within the final 218, especially the final 150, and
more especially the final 100 amino acid residues of the C-terminus
of the Hly A protein.   Thus the DNA coding for the further peptide
may comprise a sequence present within the final 654, especially the
final 450, more especially the final 339 and most especially the
final 96 nucleotide residues of the 3' terminus of an hly A coding
sequence.   For example, this DNA may comprise a sequence present
within the 654 nucleotides shown in Figure 3, preferably within the
final 450, especially within the final 339, and most especially
within the final 96 of the 3' terminus thereof.   It will be
appreciated, however, that it is the amino acid sequence coded by
this DNA sequence which is involved in secretion and thus, in view of
the redundancy of the genetic code, other DNA sequences which code

for the relevant amino acid sequence may also be used.   Furthermore,
it will be appreciated that substituted or modified analogues of
authentic haemolysin C-terminal secretion sequences may be used, and
thus the DNA sequence coding for the further peptide may include any
DNA sequence which codes for such analogue amino acid sequences.
Usually, such analogues will have at least 80%, preferably at least
90%, most preferably at least 95% homology with the corresponding
authentic haemolysin C-terminal secretion sequence.

Further, requisite DNA sequences may be wholly or partly
synthesised by oligonucleotide synthesis techniques and sequences
ligated using methods well known in the art.

Preferably the DNA sequence of the third aspect of the invention
comprises a first 5' DNA sequence coding for the polypeptide and a
second 3' DNA sequence coding for the further peptide, and especially
also an additional DNA sequence coding for a selective cleavage site
linking the 3' end of the first DNA sequence and the 5' end of the
second DNA sequence.

The DNA sequence of the third aspect of the invention may be
used in the construction of vectors, especially expression vectors,
for transformation or transfection of appropriate host cells.

Thus, in a fourth aspect, the invention provides a vector
comprising a DNA sequence according to the third aspect of the
invention.

Such vectors typically include plasmids and are
characteristically adapted for use in a given host cell type by the
provision of selectable markers, promoters, optionally suitable
replication sequences, and other control regions as appropriate.
Such vectors include bacterial, e.g. E. coli, expression vectors such
as plasmids derived from, for example, pBR322 and pAT153.   Suitable
selectable markers and promoters are well known in the art and
include antibiotic resistance markers and promoters such as the tac
promoter or the tryptophan operon promoters, e.g. the trpE promoter.

Vectors for transfection of mammalian cells may for example be
based on bacterial plasmids such as pBR322 to which one or more
suitable promoter/enhancers, selectable markers, promoters,
eukaryotic replication origins and optionally polyadenylation sites
5   have been added.

Furthermore, in a fifth aspect the invention also provides an
expression vector containing a first DNA sequence coding for a
peptide comprising a haemolysin C-terminal secretion sequence and an
unique restriction site positioned relative thereto in such a way
10   that expression of a fusion protein comprising the peptide and a
further polypeptide may be obtained when a second DNA sequence coding
for the polypeptide is inserted at the unique restriction site.

Preferably separate vectors according to the fifth aspect of the
invention are provided to accommodate the second DNA sequence in any
15   of the three reading frames relative to the reading frame of the
first DNA sequence.   Three such vector constructions are described
hereinafter by way of example.   Using such vectors DNA coding for a
desired polypeptide may be inserted at the unique restriction site to
provide an expression vector according to the fourth aspect of the
20   invention.   Vectors according to the fourth aspect of the invention
may be used to transform or transfect appropriate host cells and the
transformed host cells may be cultured to express fusion proteins
according to the second aspect of the invention.

Thus in a sixth aspect the invention further provides host cells
25   transformed with a vector according to the fourth aspect of the
invention.

Suitable host cells include eukaryotic host cells e.g. yeast and
mammalian cells and prokaryotic host cells which are typically
bacteria, preferably Gram-negative bacteria, such as E. coli.
30   Suitable mammalian hosts include, for example, chinese hamster ovary
cells and rodent myeloma cells.   It will be appreciated, however,
that in order for the haemolysin secretion pathway to operate to
secrete the fusion protein, it is necessary that the host cells also

- 10 -

express functional Hly B and D proteins.   Preferably the functional
Hly B and D proteins may be present in, or may be produced in, the
host cells in increased amounts.   These proteins may be supplied by
the host cells themselves;   for instance, Hly + host cells, e.g. E.
coli SE5000, (pLG 570) may be used.   Alternatively, the Hly B and D
proteins may be provided by co-transformation or co-transfection of
the host cells with an appropriate vector or vectors containing hly B
and hly D genes.   Such co-transformed or co-transfected hly B and
hly D may be present in the same vector as the DNA sequence coding
for the fusion protein or may be co-transformed or co-transfected in
one or more further vectors.   When the host cells are co-transformed
with hly B and hly D genes they are preferably Rec A⁻ host cells.

The methods used for transformation and transfection of host
cells and cultivation of transformed and transfected host cells are
well known in the rec DNA art.   A fusion protein comprising the
desired polypeptide, is secreted by the host cells and thus may be
advantageously recovered from the culture medium without need for
disruption of the host cells and preferably does not require
denaturation/renaturation treatment to give the polypeptide product
in soluble, native, biologically functional form.   The fusion
protein may be further processed as required;   for instance, cleaved
to yield the polypeptide product.

Brief Description of the Drawings

The invention is further described by way of illustration only
in the following non-limiting examples which refer to the
accompanying diagrams in which:

Figure 1 shows: a restriction site map of pLG570 and
                         plasmid diagram indicating the mode of
                         construction of plasmid pLG609;

Figure 2 shows: A.   Graph of growth vs time for E. coli SE5000
                         transformed with plasmid pLG609 alone (▲)
                         and co-transformed with plasmid pLG575 (●).

- 11 -

B.   SDS-PAGE gels of:

Panel B - total cell protein of <u>E. coli</u>
SE5000 (pLG609) samples taken over a
period of time, and

Panel C - samples of the culture
supernatant fractions from <u>E. coli</u> SE5000
(pLG609/pLG575) taken over a similar
period of time.

Ac identifies the 23Kd Hly A - C-terminal
peptide.

Figure 3 shows: A.   Restriction map comparison of <u>hly A</u>
between pSF4000 and pLG570.

B.   657 nucleotides of coding sequence (lower
line) of the 3' terminal of an <u>hly A</u> gene
(pLG570) and the corresponding predicted
218 amino acid sequence (upper line) of
the C-terminal of the Hly A protein.

Figure 4 shows: A graph of the hydropathic index of the
final C-terminal 82 amino acids of the
sequence of Figure 3.

Figure 5 shows: A.   Restriction map of the haemolytic
determinant carried by pLG570, and
position of Tn5 insertions.

B.   SDS-PAGE analysis of total cell protein,
obtained when pLG570:Tn 5-1 to 25 were
complemented with pLG575 to provide export
functions, followed by Western blotting
and identification of gene products
encoded by <u>hly A</u> with anti Hly A immune
serum.

Figure 6 shows: Analysis of proteins directed by pLG573
and pLG574.

- 12 -

Figure 7 shows: The nucleotide sequence in the region of
                   the inserted unique SmaI site of vector
                   constructs pLG609-1, pLG609-2 and pLG609-3.

Figure 8 shows: The construction of a lacZ-ompF-hly A gene
                   fusion.

Figure 9a shows: SDS-PAGE analysis of the secretion of the
                   23Kd C-terminal fragment of Hly A from E.
                   coli SE5000 containing pLG609 encoding the
                   3' end of Hly A and pLG575 encoding Hly B
                   and Hly D.

Figure 9b shows: SDS-PAGE analysis of cell and culture
                   supernatant samples taken from E.coli
                   JM101 (pLG632/pLG575).

Figure 9c shows: SDS-PAGE analysis of the time course of the
                   secretion of the 56Kd chimeric protein from
                   E.coli JM101 (pLG632/pLG575).

Figure 10 shows: A partial map illustrating the
                   construction of plasmids encoding various
                   segments of the C-terminus of Hly A and
                   deletion of hly A sequences from within the
                   lacZ-ompF-hly A gene fusion.

Figure 11 shows: SDS-PAGE analysis of peptides secreted into
                   the medium by E.coli JM101 (pLG575)
                   carrying 1).pLG609; 2).pLG620; or 3).pLG621.

Figure 12 shows: The sequence of the final 200 C-terminal
                   amino acids of Hly A.

Figure 13 shows: SDS-PAGE analysis of supernatant and cell
                   samples of E.coli JM101 (pLG632/pLG575),
                   E.coli JM101 (pLG633/pLG575) and E.coli
                   JM101 (pLG634/pLG575).

- 13 -

Figure 14 shows: A map of plasmid pTTQ18.

Figure 15 shows: Construction of plasmid pPH-1, carrying a
fragment of the prochymosin gene with the
hly A - C-terminal (23Kd) coding region
fused to the C-terminus.

Figure 16 shows: SDS-PAGE analysis of the production over a
period of time of the 40K hybrid
pro-chymosin-Hly encoded by pPH-1.

Figure 17 shows: pPH-1, pPH-2 and pPH-3 which contain the
E.coli haemolysin 23Kd secretion signal
available from the prochymosin (pMG168)
SmaI site in each of the three reading
frames.

Figure 18 shows:.   The construction of plasmid pApa-2,

Figure 19 shows: SDS-PAGE analysis of the production over a
period of time of the 50K hybrid encoded by
pApa-2.

Detailed Description of the Embodiments

Materials and Methods

This section contains major methods used throughout the following
examples.   Other details to be found in references cited in text.

Bacterial strains and growth conditions

Escherichia coli SE5000 rpsL, ara139, Δ(argF-lac) μ169,
recA57, MC4100 rpsL, ara139, Δ(laclPOZA) μ169, thi;   JM103
Δ(lac-pro), supE, thi, strA, endA, sbcB15, hsdR4;   F'tra D36,
proAB, lacI^Q, ZΔ M15.

Escherichia coli JM101 supE, thi Δ(lac-proAB) (F', traD36,
proAB, lacI^q ZΔM15).

- 14 -

These strains were grown with aeration at 37°C in L broth and for
plasmid-containing strains chloramphenicol (25 µg/ml) or ampicillin
(100 µg/ml) was added as appropriate.

Plasmids

Bacteriophage M13mp18 (Yanisch-Perron et al, 1985 Gene 33 103-119)

Vector plasmids pACYC184 cam^R, tet^R (Chang and Cohen, 1978 J.
Bacteriol. 134 1141-1156); pLG339, kan^R, tet^R (Stoker et al, 1982);
pUC12 amp^R, lacZ' (Norrander et al, 1983 Gene 26 101-106); pOU71
rep^{ts}, amp^R (Larsen et al, 1984 Gene 28 45-54).

The relevant Hly markers carried by different plasmids are
indicated in Table 1.

pLG570 contains a complete haemolysin determinant and purely for
convenience has been deposited at and is freely available from the
National Collections of Industrial and Marine Bacteria under Accession
No. NCIB 12466.   PLG570 has been deposited in an E. coli K12 SE5000
host.   PLG575 is a subclone from pLG570 encoding the export genes,
hly B and hly D (Mackman et al, (1985a) Mol. Gen. Genet. 201 282 -
288).   pLG609 was constructed by inserting the 3' end of hly A behind
the tac promoter of the expression vector pTTQ18 (Nicaud et al, (1986)
FEBS. Lett. 204 331 - 335).   pLG361 contains the ompF gene (Jackson
et al, (1985) EMBO. J. 4 2377 - 2383).   All the following plasmids
pLG620, pLG621, pLG631, pLG632, pLG633 and pLG634 were constructed
during these studies and are shown in Figures 8 and 10.   Plasmid
pLG603 was consructed as described in Gray et al (1986) (MGG 205:
127-133) using plasmid pLG339 (Stoker et al (1982) Gene 18 335-341).

Preparation of cell and culture supernatant samples

Cultures were grown in rich medium to A$_{450}$ = 0.4 before IPTG
(Sigma) was added to a final concentration of 50 µg/ml to derepress
the tac or lac promoters.   Cell samples were prepared by harvesting
the cells and resuspending directly in SDS-sample buffer (Laemmli,
(1970) Nature 227 680 -685).   Culture supernatant samples were
prepared by first removing the cells by centrifugation (Sorvall SS34,

- 15 -

1600 rpm 15 min) and then adding TCA to a final concentration of 10%
to the supernatant.   Precipitated proteins were harvested by
centrifugation (Sorvall HB4, 10000 rpm 10 min) and the TCA neutralised
using a saturated Tris solution before proteins were solubilised in
5   SDS-sample buffer.

Enzymes

Restriction enzymes and S1 nuclease were obtained from Bethesda
Research Laboratories, T4 ligase from Biolabs and Klenow from
Boehringer.   Reaction conditions were those described by the
10   manufacturers.

DNA fragment purification

DNA restriction endonuclease fragments were purified from agarose
gels as described by Dretzen et al, (1981) (Biochem 112 295 - 298).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

15   The basic procedure was that of Laemmli (1970)(Nature 227
680-685) using either an 18% or 11% w/v acrylamide resolving gel with
a 7% stacking gel;   acrylamide monomer : dimer ratio of 44:0.8.   Gels
were stained using Coomassie brilliant blue (0.05% w/v) in a solution
containing 10% v/v glacial acetic acid and 25% v/v propan-2-ol.

20   ## Western blotting

Electrophoretic transfer of proteins from acrylamide gels onto
nitrocellulose and subsequent immunological detection of proteins
using either rabbit anti-Hly A or rabbit anti-OmpF and horseradish
peroxidase conjugated to goat anti-rabbit IgG (Nordic Immunological)
25   was carried out as described by Towbin et al, (1979) (Proc. Natl.
Acad. Sci. USA 76 4350 - 4354) except that the substrate,

- 16 -

o-dianisidine, was replaced by 0.5 mg/ml 3, 4, 3',
4'-tetra-aminobiphenyl hydrochloride.

Haemolytic Assay

5   Values for haemolytic activity can vary widely according to the
particular Hly determinant, growth media (affecting stability), the
host strain and cellular location.   Spurious results can also arise
through non-specific lysis of red cells (see below).   In addition,
release of haemoglobin is not an enzymatic process since the pore
forming haemolysin is consumed in the reaction (Bhakdi et al 1986

10   Infect. and Immun. 52 63 - 69).   For these reasons great care is
required in establishing conditions in which haemolytic activity is
directly proportional to the level of toxin present in different
samples.   Thus, fresh sheep erythrocytes (Oxoid) were always added
in excess for each haemolysin sample i.e. conditions where the

15   reaction (release of haemoglobin) followed linear kinetics over a
selected time period.   These optimal conditions were established by
carrying out a preliminary time course experiment with a range of
sample volumes.   Individual bio-assays were then conducted as
follows: duplicate sample vol. (5-50 μl) were mixed with washed

20   erythrocytes in buffer (Mackman and Holland, 1984a MGG 193 312-315) to
a concentration of 10% v/v erythrocytes in a final volume of 1 ml.
These were incubated for 10 or 20 min respectively and then the
remaining intact erythrocytes removed immediately by centrifugation.
700 μl of the supernatants from each sample were taken for

25   measurement of free haemoglobin at A_{543} in a spectrophotometer.   The
results for the 10 and 20 min incubations were used to confirm the
linearity of the bio-assay and the average value taken.

1 unit of haemolytic activity was defined as the amount of toxin
required to release 50 mg haemoglobin in 1h per 1 ml of sample at

30   37°C.   In the case of periplasmic samples, in which activity is
usually extremely low or undetectable, the presence of 5 mM $MgCl_2$ in
the buffer can cause spurious lysis of erythrocytes following
centrifugation of the cells prior to measuring haemoglobin in the
supernatant.   This was avoided by removing the pelleted cells

- 17 -

immediately from the supernatant fraction.   In general, in order to
minimise variation in haemolytic activities these studies where
possible involved isogenic strain backgrounds and the samples for
analysis were obtained from exponentially growing cells

5      $(A^{450} = 0.9)$ in nutrient broth (Oxoid) plus 10 mM $CaCl_2$.   Under these
conditions haemolytic values in the medium are approaching the maximum
(Nicaud et al, 1985a MGG 199 111-116).   Finally, we observed that as
cells approach the stationary phase the distribution of haemolysin
changes leading to a slightly greater intracellular accumulation,

10   although periplasmic values are still very low.

Detection of Hly A and Hly A truncates with antibody

As described by Towbin et al (1979 J. loc cit) proteins were
separated by SDS-PAGE and then transferred to nitrocellulose by
Western blotting.   The Hly A derivatives were immunologically

15   detected using anti-haemolysin antibody (Mackman et al 1985a MGG 201
282-288) followed by treatment with peroxidase-conjugated antibody.

Cell fractionation

Cells were grown in nutrient broth supplemented with 10 mM
$CaCl_2$.   Periplasmic fractions were released by osmotic shock as

20   described by Nossal and Heppel (1966 J. Biol. Chem. 241 3055-3062);
sonication of the spheroplasts then led to the release of the
cytoplasmic fraction.   The relative contamination of fractions was
monitored by assaying a whole cell sample and all individual cell
fractions and washes for both malate dehydrogenase (MDH-cytoplasm) and

25   β-lactamase activities (BLA-periplasm).   MDH was assayed by addition
of oxaloacetic acid and NADH, and monitoring the reduction in
concentration of NADH.   BLA was assayed as described by O'Callaghan
et al (1972 Antimicrobial Agents and Chemotherapy 1 283-288).
Fractions were only taken for further analysis when the cross

30   contamination was less than 10%.   In all cases activity in the
fractions was greater than 95% of the whole cell sample.

- 18 -

Attempts to remove membranes from the cytoplasmic fraction by
centrifugation resulted in complete and irreversible loss of
haemolytic activity from both fractions during the procedure.   All
cytoplasmic fractions indicated therefore still contain cell membranes.

5   DNA sequence analysis

Restriction fragments cloned into the bacteriophage M13 mp18 were
used as template for the dideoxy chain termination technique first
described by Sanger et al (1977 Proc. Natl. Acad. Sci. 74
5463-5467).   The resulting DNA sequence was analysed for hydropathy
10   values (Kyte and Doolittle 1982 J. Mol. Biol. 157 105-132) and for the
predicted secondary structure by Garnier (Garnier et al 1978 J. Mol.
Biol. 120 97-120) analysis using programmes supplied by M. Stark
(Leicester Biocentre).

EXAMPLE 1

15   The C-terminal 23Kd peptide of E.coli Hly 2001 contains all
the information necessary for its secretion by the Hly export
machinery

The production of haemolysin by certain pathogenic strains of E.
coli is encoded by an approximately 7.5 kilobase sequence present on
20   plasmids or integrated into the chromosome (Welch et al (1981) Nature
294 665-667; Muller et al (1983) J. Bact. 153 846-851).   The Hly
determinant is composed of at least 4 genes: hly A, the structural
gene for the toxin;   hly C, required for post translational
modification of Hly A but not for its secretion;   hly B and hly D,
25   essential for export of the toxin through the envelope and into the
medium (Mackman et al (1985a) MGG 201 282-288; Nicaud et al (1985b)
FEBS Lett. 187 339-344).   Previous studies have demonstrated that hly
A encodes a 107 kilodalton polypeptide (Nicaud et al (1985a) MGG 199
111-116; Gonzalez-Carrero et al (1985) MGG 99 106-110) which is
30   exported to the medium without proteolytic processing of the
N-terminus (Felmlee et al (1985a) J. Bact. 163 88-93).

Furthermore, DNA sequence data for hly A indicates the complete
absence of an N-terminal signal sequence (Felmlee et al (1985b) J.
Bact. 163 94-105). In view of this and the apparent absence of a
periplasmic intermediate in haemolysin secretion, we have proposed a
5   model for export of the haemolysin (Mackman et al (1986) Curr. Top.
Micro Immun. 125, p159 - 181, Springer Verlag). In this we envisage
a first step involving recognition of cytoplasmic Hly A by the export
machinery located in the inner membrane followed by a second step
which results in direct extrusion of the toxin to the medium.

10   Recently, we have shown that secretion of haemolysin from E. coli
can be blocked by deletion of approximately 27 amino acids from the
C-terminus of Hly A, although this truncated molecule is still
haemolytically active. In this Example we describe additional
evidence that a secretion signal is entirely located in the C-terminus
15   of Hly A.

Construction of Plasmid pLG609 encoding a C-terminal
fragment of Hly A

This was achieved (see Figure 1) by subcloning a 1.6 kilobase
EcoRI-HindIII fragment encoding the 3'-end of hly A from pLG570 in the
20   correct reading frame for subsequent translation into the high copy
expression vector, pTTQ18. This plasmid carries a tac promoter
upstream of a multicloning and expression-site, together with the
lacI^q gene in order to ensure that expression from the cloned gene is
completely repressed in the absence of the inducer IPTG. Bagdasarian
25   M.M. et al (Gene 26 273 - 282 (1983)) describe analogous vectors to
pTTQ18 which may also be used to sub-clone the 3' end of hly A. The
resulting recombinant plasmid, pLG609, should contain a hybrid gene
with the tac promoter fused to the 3' end of hly A which should then
direct the synthesis of a 23 kilodalton polypeptide (Ac) corresponding
30   to the C-terminus of Hly A.

Growth of E. coli SE5000 (pLG609) in the presence and absence of
pLG575 (hly B, D)

The presence and orientation of the EcoRI-HindIII fragment in the

- 20 -

plasmid vector was confirmed by restriction enzyme analysis.

The growth characteristics of strain SE5000 (pLG609) were studied
in the presence or absence of pLG575 (Mackman et al (1985a) MGG 201
282-288) a plasmid carrying hly B and D, and with addition of IPTG in
5   order to induce synthesis of Ac from the tac promoter.   Figure 2a
shows that addition of IPTG to a strain carrying pLG609 with no hly
export functions resulted in rapid cessation of growth.   In contrast,
when the export functions were also present the addition
of IPTG had little effect on growth.

10   This result indicated that the intracellular accumulation of a
polypeptide, most probably Ac, in the absence of Hly export proteins
was toxic for the cell and that this effect could be alleviated by
export of the Ac protein to the medium.

The 23Kd C-terminal fragment of Hly·A is secreted

15   The protein content of the cells and medium from the experiment
shown in Figure 2a were analysed by SDS-PAGE.   First, Figure 2b shows
the induction of the synthesis of small amounts of a 23
kilodalton polypeptide in the total cell lysate of strain SE5000
carrying only pLG609 whereas no 23 kilodalton protein was detected in
20   the culture supernatant (data not shown).   In contrast, however, as
shown in Figure 2c, in the presence of the export functions
substantial quantities of a 23Kd polypeptide accumulated in the medium
after addition of IPTG.   The identity of the 23Kd protein as a
fragment of Hly A was confirmed by the use of antiserum to purified
25   haemolysin (Nicaud et al (1985b) FEBS Lett. 187 339-344) (data not
shown).   At least one other major polypeptide accumulated
intracellularly following the addition of IPTG but this protein was
produced by the vector alone, in the absence of the hly fragment (data
not shown).

30   The results presented here demonstrated unequivocally that the
C-terminal, 23000 dalton peptide of Hly A contains all the information
necessary for its own secretion.   As expected, secretion was only

- 21 -

observed in the presence of the specific haemolysin export proteins,
Hly B and Hly D, and does not require Hly C (Nicaud et al (1985b) FEBS
Lett. 187 339-344).   These results, taken together with the data in
Example 2 (see also Gray et al 1986, MGG 205 127-133) that deletion of
a small region at the C-terminus of Hly A blocks secretion, strongly
suggests that a small region close to the C-terminus contains all the
information necessary for secretion of the intact haemolysin.   Our
results are therefore consistent with a mechanism in which the
C-terminal region of Hly A specifically interacts with Hly B and/or
Hly D in order to facilitate secretion.   Preliminary studies indicate
that these latter proteins are located primarily within the inner-
membrane,
although significant levels of Hly D also fractionate with the outer
membrane (Mackman et al (1985b) MGG 201 529-536).   In the absence of
Hly B and D, the 107,000 dalton protein accumulates intracellularly
(Gray et al MGG 205 127-133 (1986).

EXAMPLE 2

Deletion of the Hly A C-terminus blocks secretion but not
haemolytic activity.

1.   Intracellular localisation of normal haemolysin in the
     presence or absence of export functions, hly B, hly D

In this study we took advantage of the availability of various
subclones of the Hly determinant 2001 (Table 1, Mackman et al 1985a
MGG 201 282-288), including those carrying one or both export genes,
to determine the effect of the presence of these genes in
complementation tests on the production and localisation of any
intracellular haemolysin.   For the purpose of this analysis each gene
was always supplied by the same vector.   Thus the source of hly C was
pUC12 (ca 60 copies);   hly B, D, pACYC184 (ca 20 copies);   hly A
pLG339 (ca 6 copies).   This ensured that the results were comparable
and the Hly C, B D proteins were present in excess.   The results of
these analyses with that for the fully reconstituted system (e.g.

– 22 –

pLG583 <u>hly A</u>, 577 <u>hly C</u>, 591 <u>hly B</u>, <u>D</u>) are indicated in Table 2.

Under conditions for secretion of Hly A to the medium a maximum
of 2% of haemolytic activity was usually found within the cells of
exponentially growing cultures.   Moreover, virtually 100% of this low
5   level activity was found within the cytoplasm with insignificant
levels in the periplasm as detected by osmotic shock.   When either
export function was absent the total production of haemolysin was
reduced five to ten-fold, suggesting some form of feedback regulation
on the synthesis or degradation of intracellular haemolysin.
10   Analysis of the haemolytic activity within different cellular
compartments in the absence of either or both export functions during
exponential growth revealed that more than 90% of the residual
activity was always present in the cytoplasm (Table 2).

- 23 -

Table 1

Recombinant plasmids and Hly markers

Plasmid	Resistance	Genes	Reference
pLG 570	amp	complete hly determinant	Mackman and Holland (1984b)
" 579	tet	hly B	Mackman et al (1985a)
" 575	cam	hly B hly D	" " "
" 577	tet	hly C hly A'''	" " "
" 573	cam	hly A'	" " "
" 574	cam	hly A''	" " "
" 583	kan	hly A	" " "
" 603	kan	hly A'	Gray et al (1986)
" 594	cam	hly D	Mackman et al (1985b)
" 591	amp	hly C	Nicaud et al (1985b)
hly A'	=	hly A/tet fusion resulting in 107 Kd polypeptide	
hly A''	=	" " " " " 104 Kd polypeptide	
hly A'''	=	hly A truncate resulting from termination of the gene at the EcoRI site	

Table 2

Cellular fractionation of haemolytic activity

Plasmids	Hly functions	Medium	Periplasm	Cytoplasm
1. None	None	<0.01	<0.01	<0.01
2. pLG591, 583, 575	CABD	15.4	<0.01	0.3
3. pLG591, 583	CA—	<0.01	0.2[a]	2.4
4. " ", 579	CAB-	<0.01	0.1[a]	1.5
5. " ", 594	CA-D	<0.01	0.1[a]	1.5
6. pLG570:Tn5-2, 573, 579	CA'BD*	0.8	3.1[b]	
7. " " 574, "	CA"BD	0.2	2.5[b]	
8. pLG577, 603, 575	CA'BD	0.84	0.01	1.37

* Symbols A', A'', as in Table 1.

[a] in this case level of contamination of the periplasmic fraction with cytoplasmic malate dehydrogenase reached 10%.

[b] intracellular activity.

- 25 -

2.   Effect of Tn5 insertions into hly A on secretion of the 107Kd
     protein

     A family of deletions of the C-terminus of Hly A was isolated by
insertional mutagenesis with Tn5 as described previously (Mackman and
Holland, 1984b MGG 196 129-134).   The position of the Tn5 insertions
was mapped as shown in Figure 5a.   In all cases tested Tn5 insertion
into hly A resulted in the absence of both extracellular and
intracellular haemolytic activity (data not shown).   However, when
total cell proteins were analysed by SDS-PAGE followed by Western
blotting and identification of gene products encoded by hly A with
anti Hly A immune serum, truncated derivatives of Hly A were
detected.   In contrast, no cross reacting material was detected in
the medium (Figure 5b).   The tracks of the gel shown in Figure 5b
are as follows:

          Tracks 1-5 whole cell lysates;   Track 1 Tn5-12;   Track 2
          Tn5-14;   Track 3 Tn5-16;   Track 4 Tn5-18;   Track 5 Tn5-20;
          Tracks 6-12 Culture Supernatants;   Track 6 Tn5-12;   Track 7
          Tn5-14;   Track 8 Tn5-16;   Track 9 Tn5-18;   Track 10 Tn5-20;
          Track 11 Tn5-21;   Track 12 Tn5-25;   Tracks 13 & 14 products of
          in vitro transcription translation programmed by pLG573 and
          pLG574 respectively.

     These results indicated that removal of a large portion of the
C-terminus of Hly A always resulted not only in the loss of activity,
but also in the ability of the remaining part of the molecule to be
secreted.

     The data in Figure 5, also shows that several insertions close
to the 3'-terminus of hly A resulted in truncated polypeptides all
with a molecular weight of about 80 Kd.   This suggested that such
insertions result in the formation of abnormal polypeptides which are
all proteolytically digested back to a protease resistant domain of
about 80 Kd.   In view of this phenomenon an alternative source of a
specific C-terminal deletion of Hly A was sought.

- 26 -

3. Isolation of a subclone lacking the 3' end of hly A

Previously (Mackman et al 1985a MGG 201 282-288), we have
described the construction of a plasmid, pLG574 carrying a 3 kb
BamHI-BglII fragment which appeared to encode a slightly truncated
5   form of Hly A in vitro.   Thus, unlike the Hly A deletions described
above, this plasmid encoded a polypeptide close to normal size which
was nevertheless stable.   We then examined the size of the truncated
form of the toxin produced by pLG594.   An additional subclone
pLG573, carrying the 3Kb BamHI-BglII fragment in the opposite
10   orientation (Mackman et al 1985a), was also included in the analysis.

As shown in Figure 5b (track 14), pLG574 encodes the synthesis
of an approximately 104 Kd polypeptide in vitro.   PLG573 directed
the synthesis of a polypeptide apparently indistinguishable in
mobility in SDS-PAGE from the Hly A protein (107 Kd) encoded by the
15   intact determinant in pLG570.   In fact, inspection of the downstream
sequences within the tet gene (Peden, 1983 Gene 22 277-280) in either
orientation predicts in frame fusions which would add 37 and 17 amino
acids to the Hly A protein encoded by pLG573 and pLG574 respectively
(see Figure 4).   This would result in the synthesis of proteins with
20   molecular weights similar to those observed in Figure 5.   The
alternative possibility that the original hly A sequence contains two
closely linked BglII sites close to the 3' end was eliminated by
sequencing this region of DNA (Figure 3).

4. Properties of Hly A proteins encoded by pLG573 and pLG574

25   Exponentially growing cultures of strain MC4100 carrying pLG573
or pLG574 together with an additional plasmid pLG570::Tn5-2 and
pLG579 providing hly C, D and hly B respectively in trans were
analysed for the presence of haemolytic activity.   Alternatively,
plasmids pLG577 and pLG594 may be used as a source of hly C and
30   hly D respectively.   The results in Table 2 (line 6, 7) clearly
demonstrated that strains carrying either pLG573 or pLG574 produced
only low levels of extracellular activity.   Moreover, in both cases
there was now several fold more haemolytic activity inside the cells

compared to that in the medium.   Furthermore, on sheep blood plates,
which we have found forms an extremely sensitive test for secretion,
the presence of pLG574 in bacterial colonies failed to promote any
detectable release of haemolysin whilst pLG573 gave only very small

5   haloes (data not shown).   These results strongly suggested that the
truncated forms of Hly A produced from pLG573 and pLG574 were
defective in secretion.   In order to confirm this effect under
conditions comparable to those described in the first section above,
the BamHI-BglII fragment encoding hly A in pLG573

10   was transferred to the low copy number vector, pLG339, generating the
recombinant plasmid, pLG603 (see Figure 5a).   Production of   ·.
haemolytic activity directed by this plasmid was measured and once
more we observed a greater than 10-20 fold reduction in the
extracellular level of haemolysin (Table 2, line 8) compared to

15   comparable constructions encoding the intact Hly A.

       The levels and localisation of Hly A protein, directed by pLG573
       and pLG574 in the presence of the export functions were also analysed
       by immunoblotting using antibody to Hly A.

       Figure 6 shows proteins separated by SDS-PAGE transferred by
20   Western blotting to nitrocellulose and visualised using
       anti-haemolysin antibody followed by staining with perioxidase
       conjugated antibody.   pLG573 and pLG574 were complemented with
       pLG579 and pLG570:Tn5-2 providing hly B and hly C, D
       respectively in trans.   Tracks 1-2 whole cell lysates.   Track
25   1 pLG574; Track 2 pLG573.   Tracks 3-6 culture supernatants.
       Track 3 pLG574; Track 4 pLG573; Track 5 E.coli culture
       supernatant no plasmid; Track 6 authentic Hly A (107K) from
       pLG570.

       As shown in Figure 6 insignificant amounts of Hly A were
30   detected in the medium with either pLG574 (track 3) or pLG573
       (track 4).   However, intracellular levels of 107 Kd polypeptide were
       readily detected in both cases (Figure 6, track 1, 2).

- 28 -

5.   DNA sequence of the 3' end of hly A 2001

Since the C-terminal region of Hly A is clearly involved in the
secretion process it was necessary to obtain the DNA sequence of the
corresponding region of hly A.   The results of the DNA sequence
analysis and the predicted amino acid sequence for the last 218
residues compared with those of Hly psF4000 described by Felmlee et
al 1985b (J. Bacteriol. 163 94-105), are presented in Figure 3b.

The overall sequence in this region is highly conserved, with
95% homology at the DNA level, and 94% homology at the amino acid
level.   The sequence data and restriction enzyme analysis also
confirmed the restriction enzyme site differences between Hly 2001
and the sequence of Felmlee et al.   This included the surprising
finding that the BglII site in the latter sequence has been replaced
by a quite different BglII site (Figure 3a).   This BglII site in the
Hly A 2001 sequence is extremly close to the terminus and enabled us
to generate the 27 amino acid deletion of Hly A described above.

From the Hly A 2001 sequence data and knowledge of the down
stream sequences at the cloning site junctions a profile of the
hydropathy index for the terminal 82 amino acids of Hly A and for the
deletions in pLG574 and pLG573 generated by cleavage at the BglII
site was obtained (Figure 4).   In addition to the likely structure
of the terminal regions formed in pLG573 and pLG574 the figure
indicates the position of the BglII site which coincides with a rare
feature in the predicted secondary structure of Hly A, a short ∝
helical region.   This feature is conserved in HlySF4000 as is a
stretch of 13 relatively hydrophobic amino acids (residues 191-203)
overlapping the predicted ∝ helix between residues 185-196.

EXAMPLE 3

Subclones of pLG609 using EcoR1 linker DNA to generate different
reading frames in the Hly, 23Kd secretion signal region

Plasmid pLG609, derived as described in Example 1, was digested

with the restriction endonuclease EcoRl.   Three synthetic
oligonucleotides:

1). GAATTTTCCCCGGGGAA

2). GAATTTTCCCGGGAA

3). GAATTTCCCGGGA

containing a Smal recognition site were synthesised using methods
well known in the art and ligated with the digested pLG609 to yield
novel vectors pLG609-1, pLG609-2 and pLG609-3 respectively as
described in Figure 7, each containing a different reading frame for
the Hly A 23Kd secretion signal region.

The vectors pLG609-1, pLG609-2 and pLG609-3 thereby accommodate
the insertion of a second DNA sequence in any of the three reading
frames relative to and upstream of the 3' end of hly A.

EXAMPLE 4

Secretion of a bacterial outer membrane protein fused
to the Hly A C-terminal region

A.   Construction of a lacZ-ompF-hly A chimeric gene

A chimeric protein containing at the N-terminus 10 amino acids
of a β-galactosidase moiety with its translational start signals, a
central portion containing the majority of the outer membrane porin
OmpF and its C-terminus the secretion signal of Hly A was
constructed. As a first step in this construction a lacZ-ompF
hybrid gene was made in pUC12 (Figure 8). The core of the ompF
gene, lacking DNA sequences encoding the first 11 amino acids of the
N-terminus and the final 30 amino acids of the C-terminus of mature
OmpF was removed from pLG361 (Jackson et al 1985 EMBO J. 4 2377-2383)
on a 0.9 kb BglII-HincII fragment. This was inserted downstream of
the lac promoter of pUC12 which had been digested with BamHI and
HincII. The intermediate plasmid pLG631 encoded a chimeric protein

containing at its N-terminus the first 10 amino acids of a
β-galactosidase moiety fused to the central 300 amino acids of
OmpF.   Secondly, pLG609 was digested with EcoRI and filled in using
Klenow to make blunt ends, before being further digested with HindIII
5   to generate a 1.6 kb fragment.   This contained the 3' end of hly A
and was inserted into pLG631, which had been digested with HincII and
HindIII.   The resulting plasmid pLG632 was confirmed by restriction
analysis and contained a lacZ-ompF-hly A gene fusion under the
control of the lac promoter.   Thus, pLG632 was predicted to encode a
10   56 Kd chimeric protein including the final 218 amino acids of Hly
A.   Transcription of this gene fusion was under the control of the
lac promoter and translation was controlled by the start signals of
β-galactosidase.

B.   Secretion of the 56 Kd chimeric protein into the medium
15   is dependent upon the presence of Hly B and Hly D

The 23 Kd C-terminal peptide of Hly A is specifically secreted
into the medium from E. coli SE5000 containing the export functions,
Hly B and Hly D, (Nicaud et al 1986b FEBS Lett. 204 331-335) encoded
by pLG575 (Mackman et al J. Loc. Cit. 1985a).   Therefore, we used
20   the same conditions in order to test whether the 56 Kd chimeric
protein, including this 23 Kd peptide of Hly A, could be recognised
and specifically released into the medium using this system.   For
comparison, Figure 9a shows the rapid accumulation of the 23 Kd
peptide in the medium of strain SE5000 (pLG575) after derepression of
25   the tac promoter of pLG609 with IPTG.

Proteins were analysed by SDS PAGE and visualised by Coomassie
brilliant blue (top panel) or by using polyclonal antibody to
Hly A in a Western blot (bottom panel).   IPTG was added at
t=0h. Cells: track 1, t=0h;   track 2, t=2h;   track 3, t=3h;
30   track 4, t=4h.   Supernatant: track 1, t=0h;   track 2, t=2h;
track 3, t=3h;   track 4, t=4h.

A Western blot (bottom panel) further shows that the vast
majority of the 23 Kd protein is indeed in the medium and does not

accumulate significantly inside the cells.　pLG632 was transformed into strain JM101 F'__lacI__^{q} and the __lac__ promoter, upstream of the __lacZ__-__ompF__-__hly A__ hybrid gene, was derepressed using IPTG.　Figure 9b shows the induction of the 56 Kd chimeric protein, indentified in a Western blot using polyclonal antibody to Hly A, in the presence and absence of the haemolysin export genes.

　　Cell and culture supernatant (SN) samples were taken from cultures of __E.coli__ JM101 (pLG632) and __E. coli__ JM101 (pLG632/pLG575) (referred to as -export and +export respectively) at the time of induction with IPTG and 2h post induction.　Proteins were analysed by SDS PAGE and visualised using polyclonal antibody to Hly A.　The arrow indicates the position of the 56Kd ompF-hlyAc hybrid.　-export: track 1, t=0h (cells);　track 2, t=0h (SN);　track 3, t=2h (cells);　track 4, t=2h (SN), +export: track 5, t=0h (cells);　track 6, t=0h (SN);　track 7, t=2h (cells);　track 8, t=2h (SN).

　　In the absence of Hly B and Hly D, the 56 Kd protein remained cell associated.　However, when the haemolysin export genes were provided by pLG575, the vast majority of the chimeric protein was now detected in the culture medium, with no detectable amounts inside the cells.　Clearly, both the 23 Kd peptide of Hly A and the 56 Kd chimeric protein are specifically recognised by the haemolysin export pathway and secreted into the medium.　Figure 9c shows a time course for the accumulation of the 56 Kd chimeric protein in the culture supernatant after induction and the stained profile indicated that this protein was specifically released into the medium.

　　A culture of JM101 (pLG632 and pLG575) in L broth was induced with IPTG to derepress the lac promoter.　Cell and culture supernatant samples were taken at time of induction and at subsequent intervals.　Proteins were analysed by SDS PAGE and visualised by Coomassie blue staining.　Molecular weights are indicated in kilodaltons.　Cell samples: Track 1, t=0h;　Track 2, t=60 min;　Track 3, t=120 min;　Track 4, t=180 min;　Supernatants:　Track 5, t=0h;　Track 6, t=60 min;　Track 7, t=120 min;　Track 8, t=180 min.

Although the total amount of protein was less than the 23 Kd
C-terminal peptide alone (Figure 9a), this may simply reflect a
higher level of transcription from the tac promoter of pLG609 after
induction compared with that of the lac promoter present upstream of
5   the lacZ-ompF-hly A gene fusion on pLG632.

EXAMPLE 5

The Hly A secretion signal is located within the final 113
amino acids of the C-terminus

     The release of the 23 Kd C-terminal peptide of Hly A indicated
10   that the secretion signal was located within these last 218
     residues.   Furthermore, deletion of the final 27 amino acids
     apparently disrupted the signal and abolished secretion of the
     resulting polypeptide (Gray et al (1986) Mol. Gen. Genet. 205 127 -
     133).   Therefore, to more specifically localise the region that was
15   necessary for secretion, two plasmids were constructed which
     expressed smaller C-terminal fragments of Hly A (Figure 10).   First,
     a 138 bp DNA fragment encoding 46 amino acids was deleted from within
     hly A encoded by pLG609.   The resulting plasmid, pLG620, contained
     part of the 3' end of hly A under the control of the tac promoter and
20   expressed an 18 Kd C-terminal fragment of Hly A.   Secondly, pLG621
     was made by inserting a 353 bp DraI DNA fragment from pLG620 into the
     vector pUC12 (see Figure 10).   This created a hybrid protein
     consisting of the first 9 residues of a β-galactosidase moiety fused
     to the final 113 amino acids of Hly A.
25   This gene fusion was under the control of the lac promoter.

     In order to test whether the C-terminal peptides encoded by
     pLG620 and pLG621 were secreted by the haemolysin export pathway,
     these plasmids were transformed into JM101 containing pLG575.
     Figure 11 shows

The secretion of various C-terminal fragments of Hly A using the
haemolysin export pathway.
Proteins from culture supernatant (SN) samples were analysed by
SDS-PAGE followed by staining with Coomassie brilliant blue.
5   Track 1, SN E.coli JM101 (pLG609/pLG575) Track 2, SN E.coli
JM101 (pLG620/pLG575) Track 3, SN E.coli JM101 (pLG621/pLG575)
A 14.3Kd M.wt standard is also shown.

·The results show that upon induction both the 18 and 12 Kd
peptides encoded by pLG620 and pLG621 respectively were secreted into
10   the medium.   This strongly suggested that the secretion signal of.
the Hly A protein was contained within the final 113 amino acids of
the C-terminus.   The amount of 12 Kd peptide secreted into the
medium was lower than the amount of both 23 and 18 Kd proteins but
again this may reflect a lower level of transcription from the lac
15   promoter.

                              EXAMPLE 6

The final 27 amino acids of haemolysin can allow the
secretion of a chimeric protein into the medium

                Further information on the minimal sequence containing the Hly A
20   secretion signal was gained by studying the release of chimeric
proteins.   We have shown that the final 218 amino acids of Hly A can
direct the secretion of a 56 Kd chimeric protein composed of OmpF and
the C-terminal portion of Hly A into the medium.   We show below that
a secretion signal is located within the final 113 amino acids.
25   Thus, sequences of hly A were deleted from within the lacZ-ompF-hly A
hybrid gene, without altering the lacZ or ompF sequences, to form two
hybrid proteins which contained at their C-terminus only an
undisturbed block of the final 27 amino acids of Hly A.

                A partial restriction map of part of pLG570 is shown in Figure
30   10.   .pLG609 contains a gene fusion between lacZ and the 3' end of

hly A which encodes a 23 Kd chimeric protein consisting of 5 residues
of β-galactosidase at its N-terminus fused to the final 218 amino
acids of Hly A at its C-terminus (Nicaud et al, (1986b) FEBS Lett.
204 331-335).   Transcription of this lacZ-hly A hybrid gene is

5   controlled by a tac promoter.   pLG620 was made from pLG609 by
deleting a 138 bp BalI fragment from within the 3' end of hly A.
pLG620 contains a lacZ-hly A hybrid gene, under the control of the
tac promoter, which encodes an 18 Kd chimeric protein including 172
residues of Hly A.   pLG621 was constructed by inserting a 353

10   bp DraI fragment, isolated from pLG620, into the SmaI site of the
vector pUC12.   Recombinants were identified as white colonies by-.
growth on media containing X-gal/IPTG and the orientation of the
insert was confirmed by restriction analysis.   PLG621 contains a
lacZ-hly A gene fusion, under the control of the lac promoter, and

15   encodes a 12 Kd chimeric protein consisting of 9 residues of a
β-galactosidase moiety fused to the final 113 amino acids of Hly A.

        Figure 10 also shows a partial restriction map of part of pLG361
(Jackson et al 1985, Embo J. 4 2377-2383).   pLG632 was constructed
as previously described in Figure 8 and encodes a 56 Kd chimeric

20   protein.   pLG633 and pLG634 were both created by first digesting
pLG632 to completion with BglII and removing the sticky ends using S1
nuclease.   Secondly, the DNA was partially digested with BalI and
the large DNA fragments isolated.   After ligation, the DNA was
transformed into strain JM101 F'lacI^q and plasmids containing the

25   appropriate deletions were identified by restriction analysis.
        Restriction enzymes are as follows: B - BalI, Bg - BglII,
D - DraI, E - EcoRI.

        The two plasmids generated, pLG633 and pLG634 (Figure 10) encode
chimeric proteins of 45 and 40 Kd respectively.   Both these chimeric

30   proteins included the same 27 amino acid region from the extreme
C-terminus (see Figure 12 for amino acid residues in this region).
However, in the 45 Kd protein this small C-terminal region was
separated from the fusion site with the OmpF portion of the molecule
by 98 residues of Hly A, whereas in the 40 Kd protein only 52

35   residues of Hly A separated this region from the fusion site with
OmpF.

Secretion of the 45 and 40 Kd chimeric proteins was tested using
JM101 containing pLG575.   Cell and supernatant fractions were
Western blotted against polyclonal antibody to OmpF since antibody to
Hly A does not detect either of these proteins.   Figure 13 shows

5   that upon induction the 45 Kd chimeric protein was secreted into the
medium, although the total amount accumulated was less than that of
the 56 Kd chimeric protein and small amounts of the 45 Kd protein
were found to be cell associated.   This result suggested that the
last 27 residues of Hly A may in fact contain the majority of the

10   secretion signal and be sufficient to allow some specific secretion
through the haemolysin export pathway.   ..

As shown in Figure 13 strains E.coli JM101 (pLG632/pLG575),
E.coli (pLG633/pLG575) and E.coli JM101 (pLG634/pLG575) were induced
with IPTG and 2h after induction cell and supernatant (SN) samples

15   were analysed by SDS-PAGE and proteins detected by Western
blotting using polyclonal antibody to OmpF.Authentic OmpF is
indicated at 36Kd.   Track 1, JM101 cells;   track 2, JM101
(pLG634/pLG575) cells;   track 3, JM101 (pLG633/pLG575) cells;   track
4, JM101 (pLG632/pLG575) cells;   track 5, JM101 (pLG634/pLG575) SN;

20   track 6, JM101 (pLG633/pLG575) SN;   track 7, JM101 (pLG632/pLG575) SN.

. We have examined the final 200 C-terminal amino acids of Hly A
and their predicted secondary structure (Figure 12).

The last 200 amino acids encoded by the 3' end of hly A from
pLG570 (Gray et al, (1986) Mol. Gen. Genet. 205 127-133).   Open and

25   closed boxed areas indicate predicted regions of weak or stronger
α-helical regions respectively.

Interestingly, a potential α-helix was found between residues
167 and 177, a region spanning the BglII site used in the
construction of pLG633 and pLG634.   This region may serve to

30   separate the proposed 27 amino acid secretion signal from the
remainder of the molecule.

Significantly, by examining the fusion sites within the hly A
sequences of pLG633 and pLG634, we can see that this potential

α-helix is partly recreated in the 45 Kd chimeric protein but not in
the 40 Kd chimeric protein.   This could explain why significant
amounts of the 45 Kd protein are secreted into the medium.

Since the normal Hly secretion mechanism must be post
translational it is important that the secretion signal is not buried
within the tertiary structure of the polypeptide being transported.
Inspection of the predicted secondary structure of the normal Hly A
molecule, the smaller truncates of Hly A which are secreted, and the
OmpF and prochymosin hybrids indicate that the region of the signal
sequence always appears to form a distinct domain of secondary
structure separate from the bulk of the molecule.   It will be
appreciated therefore that although the discrete signal in the
C-terminal region of Hly A is sufficient for recognition of the Hly
B, D export system, the efficient secretion of particular hybrids may
require the insertion of spacer amino acids sufficient to ensure that
the signal sequence remains accessible to the Hly B, D secretion
machinery.

The use of antibody to OmpF also revealed in Figure 13 that
mature OmpF (36 Kd) was present in the medium in small amounts.

Mug-Opstelten and Witholt (1978 Biochim. Biophys. Acta. 508 287
- 291) have previously shown that mature OmpF protein is normally
released from wild type E. coli into the medium in a vesicle
associated form.   To test whether the 56 Kd chimeric protein was
released in the form of vesicles, culture supernatant samples were
treated with protease.   The 56 Kd hybrid protein was found to be
completely degraded by protease whereas the mature OmpF protein,
embedded in the membrane of the vesicle, was completely resistant
(unpublished data).   Moreover, when culture supernatants were
sedimented (40rpm, 4h, 50Ti rotor) the results clearly showed that
all the wild type OmpF protein was in the pellet whereas all of the
chimeric protein remained in the supernatant (unpublished data).
Therefore, secretion of the chimeric protein appears to be specific
and does not involve preferential release of portions of the outer
membrane.

EXAMPLE 7

Construction and Secretion of Prochymosin-Hly A hybrids

The EcoRl-HindIII fragment (Figure 1) carrying the 3' end of Hly
A encoding a 23 Kd protein was cloned into pTTQ18 (Figure 14) to
5   generate the plasmid pLG609 (Figure 1).   A series of subclones were
constructed by inserting various oligonucleotides into the unique
EcoRl site of pLG609 to produce different reading frames for the 3'
Hly A gene (Figure 7).

The first prochymosin-Hly A hybrid (to the XmaI site) was
10   constructed as shown in Figure 15 from the dual origin vector pMG168
(UK Patent No. GB 2136814B) and pLG609-1 to form pPH-1.   The 40 Kd
product, confirmed by antibody to Hly A was secreted into the medium
if the cells contained pLG575 carrying the export genes hly B and hly
D.   Figure 16 ·shows the time course of production of 40K hybrid
15   prochymosin – Hly encoded by pPH-1.   To induce high level expression
from the trp promoter to transcribe the hybrid gene the culture was
shifted to 37°C.   This inactivates the λ repressor CI857 leading to
extensive transcription of the λ promoter pR which in turn disrupts
downstream control of copy number of the vector.   Amplification of
20   plasmid number titrates out the trp repressor and then allows high
level expression from trp.

In Figure 16 –
Track 1 – 0 min, Track 2 – 30 min induction, Track 3 – 60 min
induction, Track 4 – 90 min induction, Track 5 – 120 min
25   induction.

In order to construct a larger portion of prochymosin (to the
ApaI site) fused to the Hly A secretion signal advantage was taken of
the availability of the Hly A secretion signal in all three reading
frames provided in the pPH plasmid series (Figure 17).   The larger
30   prochymosin hybrid was then constructed from the dual origin vector
(pMG168) and pPH-2.   The resulting plasmid, encoding a 50 Kd hybrid
protein, is pApa-2 which encodes a larger fragment of the prochymosin

gene fused to hly A - C-terminal DNA encoding the 23Kd protein (Figure 18).

Figure 19 shows the time course of production of the 50K hybrid prochymosin - Hly encoded by pApa-2.   Details as described above for Figure 16.

The results indicate that this hybrid is secreted provided the export plasmid pLG575 is also present.   The identity of the hybrid was confirmed using antiserum to both prochymosin and Hly A.

CLAIMS

1.   A process for the production of a polypeptide, in which host cells transformed or transfected with DNA coding for a fusion protein comprising the polypeptide and further peptide comprising a C-terminal secretion sequence are cultured to express and secrete the fusion protein therefrom.

2.   A process according to Claim 1 wherein following secretion, the fusion protein is cleaved to yield the polypeptide.

3.   A process according to Claim 1 or Claim 2 wherein the C-terminal secretion sequence is a haemolysin C-terminal secretion sequence.

4.   A fusion protein comprising a first polypeptide and a further peptide in which the further peptide comprises a C-terminal secretion sequence.

5.   A fusion protein according to Claim 4 wherein the fusion protein is a C-terminal fusion protein.

6.   A fusion protein according to Claim 4 or Claim 5 wherein the further peptide comprises a haemolysin C-terminal secretion sequence.

7.   A DNA sequence coding for a fusion protein according to Claim 4, Claim 5, or Claim 6.

8.   A vector comprising a DNA sequence according to Claim 7.

9.   An expression vector containing a first DNA sequence coding for a peptide comprising a haemolysin C-terminal secretion sequence and a unique restriction site positioned relative thereto in such a way that expression of a fusion protein comprising the peptide and a further peptide may be obtained when a second DNA sequence coding for the polypeptide is inserted at the unique restriction site.

10.   Host cells transformed or transfected with a vector according to Claim 8.
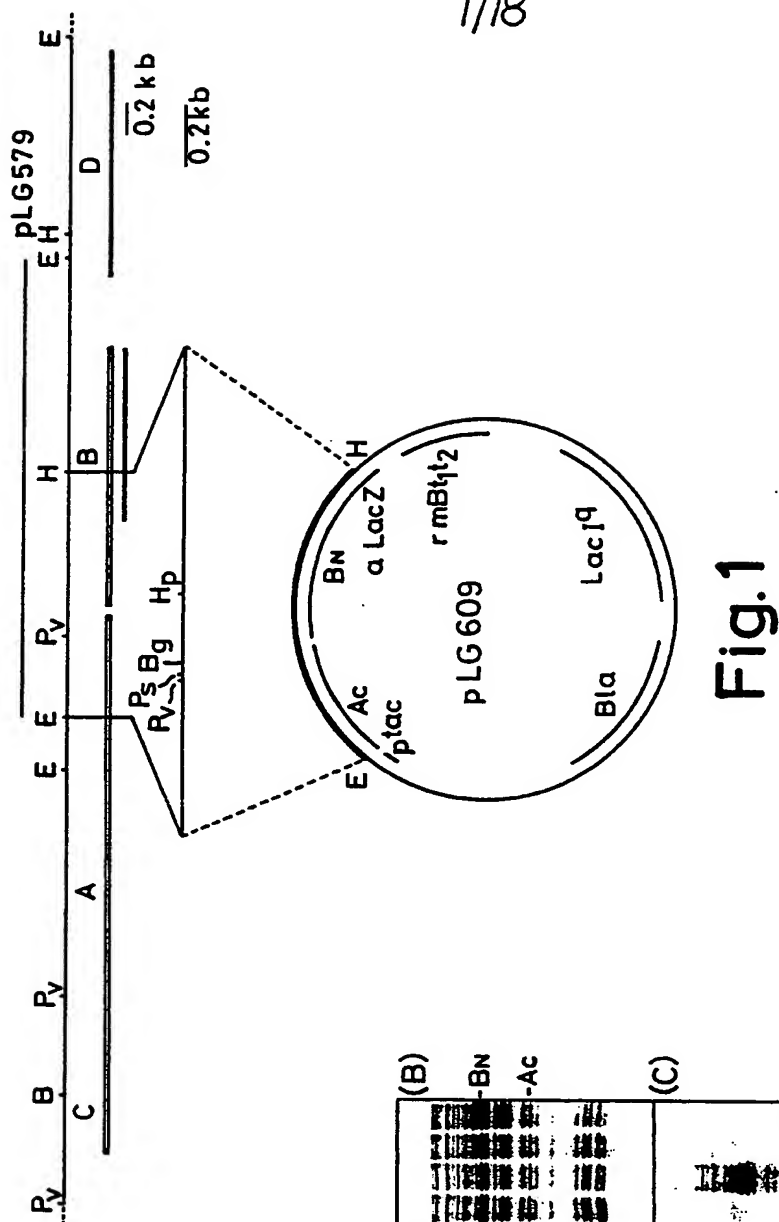
Fig. 1
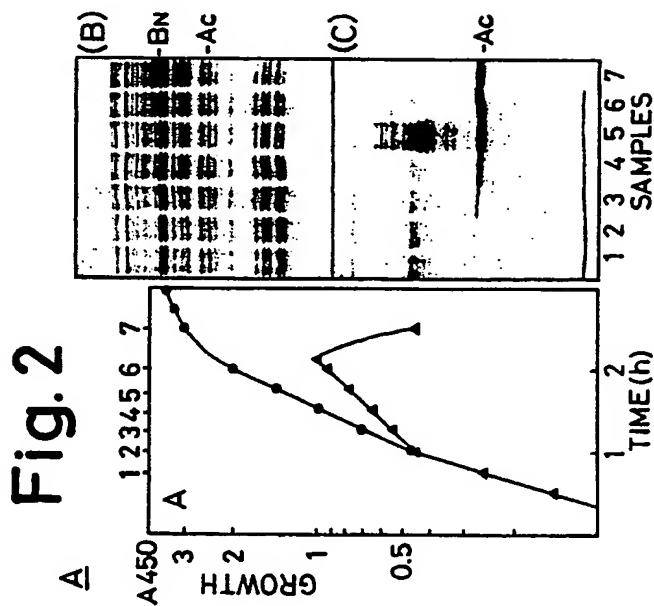
Fig. 2

Fig. 3

```
                                     10                                                    20
B  Gly Asn Ser Leu Ala Lys Asn Val Leu Ser Gly Gly Lys Gly Asn Asp Lys Leu Tyr Gly
   GGG AAT TCT CTT GCT AAA AAT GTA TTA TCC GGT GGA AAA GGT AAT GAC AAG TTG TAC GGC
   A               A                                                   C


                                     30                                                    40
   Ser Glu Gly Ala Asp Leu Leu Asp Gly Gly Glu Gly Asn Asp Leu Leu Lys Gly Gly Tyr
   AGT GAG GGA GCA GAC CTG CTT GAT GGC GGA GAA GGG AAT GAT CTT CTG AAA GGT GGA TAT
                       T


                                     50                                            Asp   60
   Gly Asn Asp Ile Tyr Arg Tyr Leu Ser Gly Tyr Gly His His Ile Ile Asp Asp Glu Gly
   GGT AAT GAT ATT TAT CGT TAT CTT TCA GGA TAT GGC CAT CAT ATT ATT GAC GAT GAA GCG
                                                                           T

            Asp                      70                                     80
   Gly Lys Glu Asp Lys Leu Ser Leu Ala Asp Ile Asp Phe Arg Asp Val Ala Phe Lys Arg
   GGG AAA GAG GAT AAA CTC AGT TTG GCT GAT ATA GAT TTC CGG GAC GTT GCC TTC AAG CGA
            C                                T         T   G


                                     90                                                   100
   Glu Gly Asn Asp Leu Ile Met Tyr Lys Ala Glu Gly Asn Val Leu Ser Ile Gly His Lys
   GAA GGG AAT GAC CTC ATT ATG TAT AAA GCT GAA GGT AAT GTT CTT TCC ATT GGC CAC AAA
       T           C


                                     110                              Ile                120
   Asn Gly Ile Thr Phe Lys Asn Trp Phe Glu Lys Glu Ser Asp Asp Leu Ser Asn His Gln
   AAT GGT ATT ACA TTT AAA AAC TGG TTT GAA AAA GAG TCA GAT GAT CTC TCT AAT CAT CAG
                                                           A           C


                                     130                                                  140
   Ile Glu Gln Ile Phe Asp Lys Asp Gly Arg Val Ile Thr Pro Asp Ser Leu Lys Lys Ala
   ATA GAG CAG ATT TTT GAT AAA GAC GGC AGG GTA ATC ACA CCA GAT TCT CTT AAA AAG GCA


   Leu                               150   Ala                                  Leu Ala
   Phe Glu Tyr Gln Gln Ser Asn Asn Lys Val Ser Tyr Val Tyr Gly His Asp Ala Ser Thr
   TTT GAA TAT CAG CAG AGT AAT AAC AAG GTA AGT TAT GTG TAT GGA CAT GAT GCA TCA ACT
   C       A                          C                   G   A           T   G C


                      Gly              170                                                 180
   Tyr Gly Ser Gln Asp Asn Leu Asn Pro Leu Ile Asn Glu Ile Ser Lys Ile Ile Ser Ala
   TAT GGG AGC CAG GAC AAT CTT AAT CCA TTA ATT AAT GAA ATC AGC AAA ATC ATT TCA GCT
       A       T
                                GT


                                     190   Ala                                            200
   Ala Gly Asn Phe Asp Val Lys Glu Glu Arg Ser Ala Ala Ser Leu Leu Gln Leu Ser Gly
   GCA GGT AAT TCG GAT GTT AAG GAG GAA AGA TCT GCC GCT TCT TTA TTG CAG TTG TCC GGT
                           A              G   A


                                     210
   Asn Ala Ser Asp Phe Ser Tyr Gly Arg Asn Ser Ile Thr Leu Thr Ala Ser Ala ***
   AAT GCC AGT GAT TTT TCA TAT GGA CGG AAC TCA ATA ACT TTG ACA GCA TCA GCA TAA
```

```
A    pSF4000 _B_____E____E____Bg_____ _
         B                                E    E    Bg
     pLG570 __B_____E__E_____Bg__
```

Fig.4

LKKAFEYQQNNNKVSYVYGHDASTYGSQDYLNPLINEISKIISAAGNLDVKEERSAASLLQLSGNASDFSYGRNSITLTASA

LKKAFEYQQNNNKVSYVYGHDASTYGSQDYLNPLINEISKIISAAGNLDVKEERSSTPDASWPASPAPQVRLLAPISPTSPMGKIGLATSCS

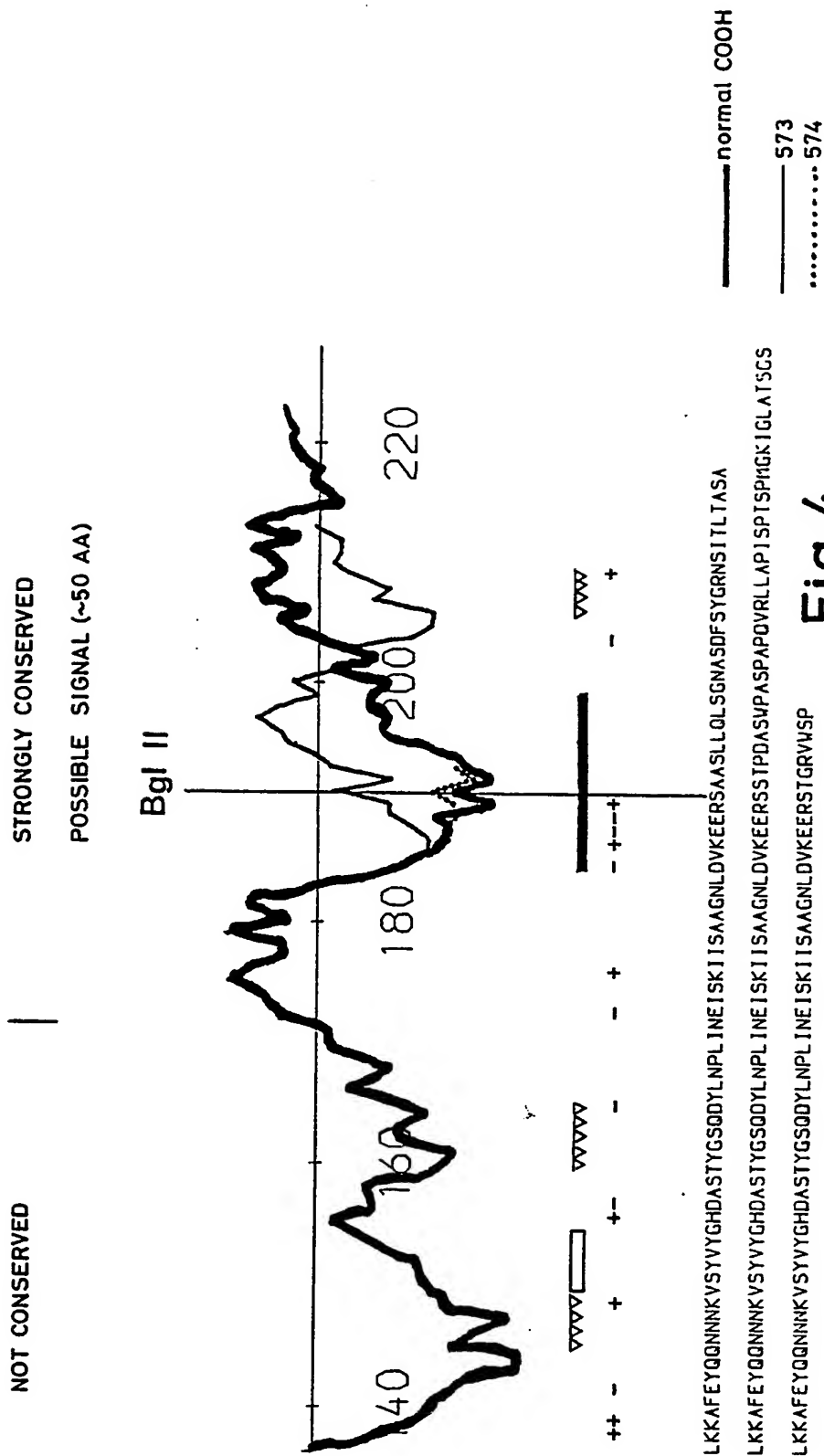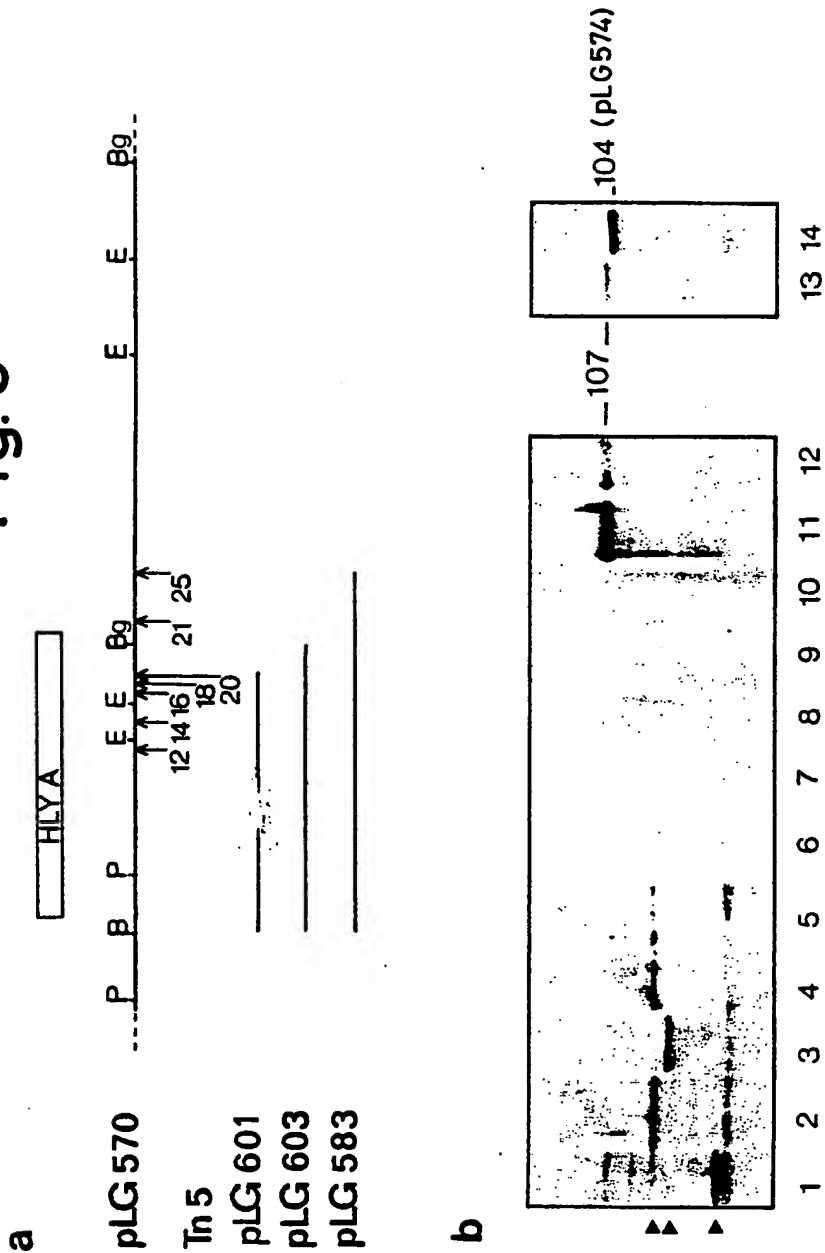LKKAFEYQQNNNKVSYVYGHDASTYGSQDYLNPLINEISKIISAAGNLDVKEERSTGRVWSP
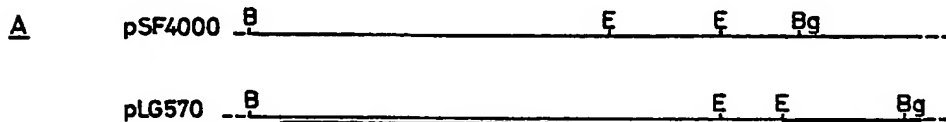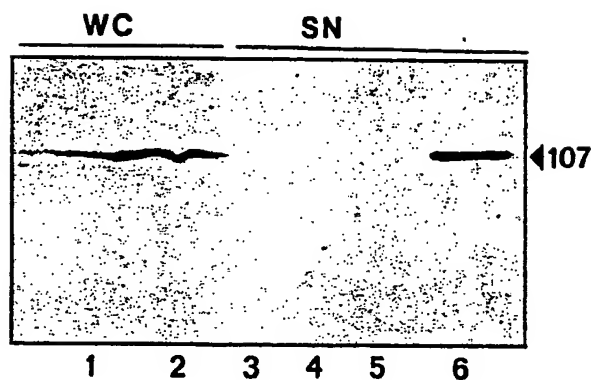
4/18

Fig. 5

5/18

Fig. 6

SUBCLONES OF PLG609, USING EcoR1 LINKER DNA, GENERATING DIFFERENT READING FRAMES TO THE HAEMOLYSIN 23KD SECRETION SIGNAL

```
                    EcoR1
PLG609          G L  AAT  TCT  CTT


                                     SMAI
PLG609 - 1      G  AAT  TTT  CCC  C | GGG  GAA  AAT  TCT  CTT


                                  SMAI
PLG609 - 2      G  AAT  TTT  CCC | GG  GAA  AAT  TCT  CTT


                              SMAI
PLG609 - 3      G  AAT  TTC  CC | G  GGA  AAT  TCT  CTT


                            BAMH1
PLG609 - 4      G  ATT  TTG  G GA  TCC  CCA  AAT  TCT  CTT
```

Fig. 7

SUBSTITUTE SHEET
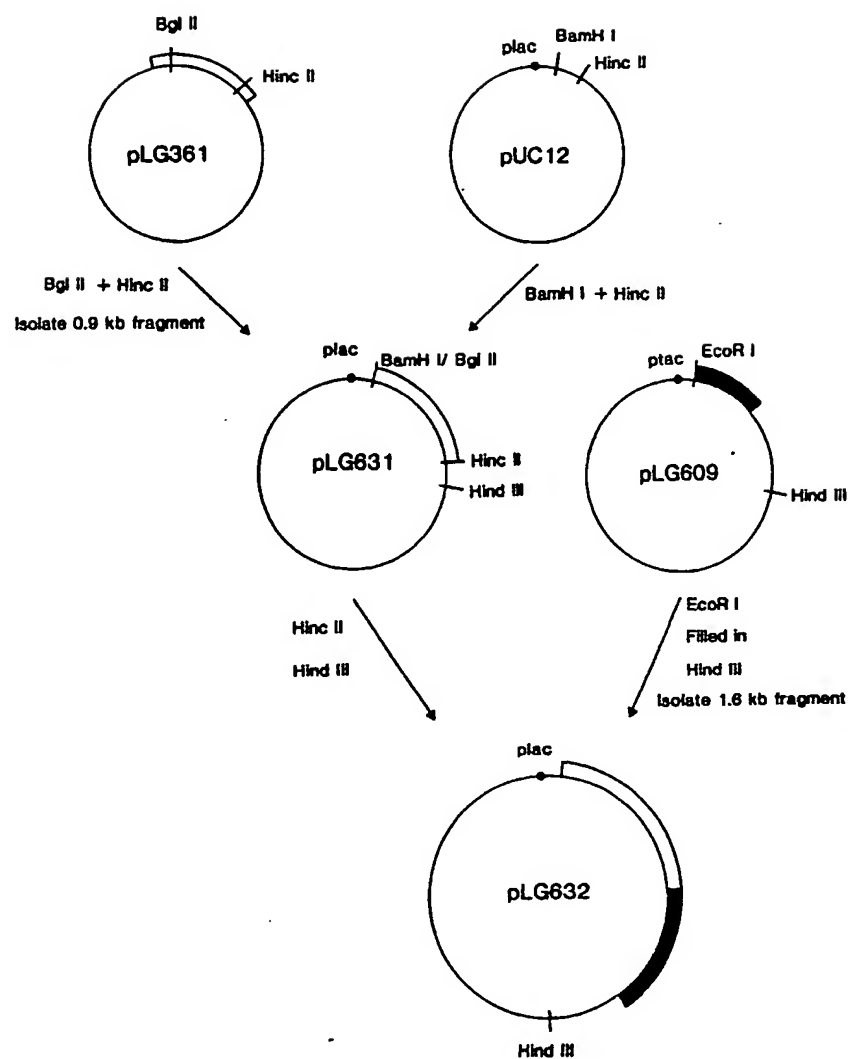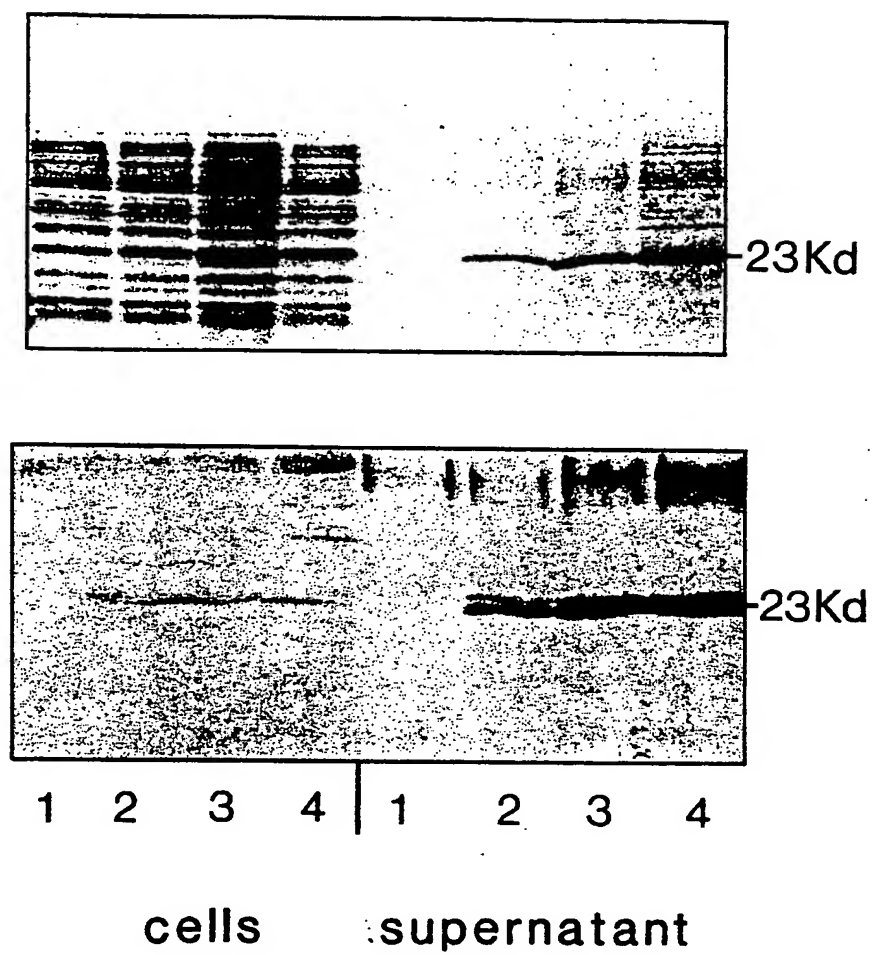
Fig. 8

1 2 3 4 | 1 2 3 4

cells   supernatant

Fig. 9a

Fig. 9b

cells   supernatant

Fig.9c

Fig.10

Fig.11

```
0                                    BALI                           50

YGSEGADLLD GGEGNDLLKG GYGNDIYRYL SGYGHHIIDD EGGKDDKLSL
      [    ]                         [       ]        [   ]
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

51                           BALI        DRAI             100

ADIDFRDVAF KREGNDLIMY KAEGNVLSIG HKNGITFKNW FEKESDDLSN
  [        ]          ████████        █████████
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

101                                                       150

HQIEQIFDKD GRVITPDSLK KAFEYQQSNN KVSYVYGHDA STYGSQDNLN
     [    ]           ████████
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

151                      BGLII                            200

PLINEISKII SAAGNFDVKE ERSAASLLQL SGNASDFSYG RNSITLTASA
     [     ]████████████████
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
```
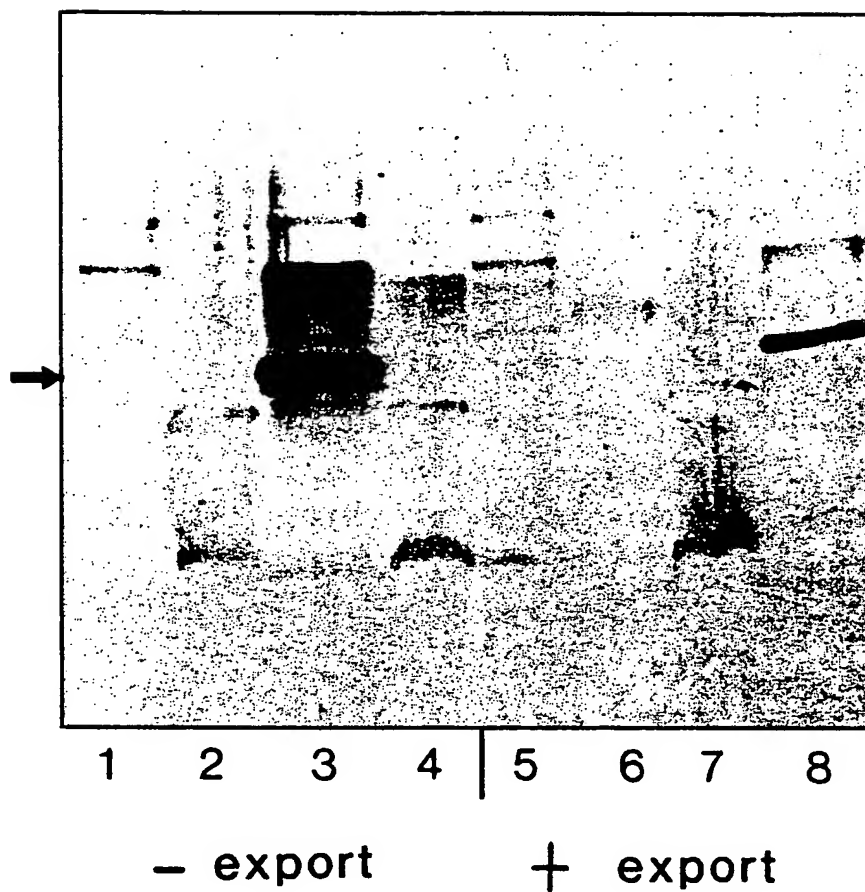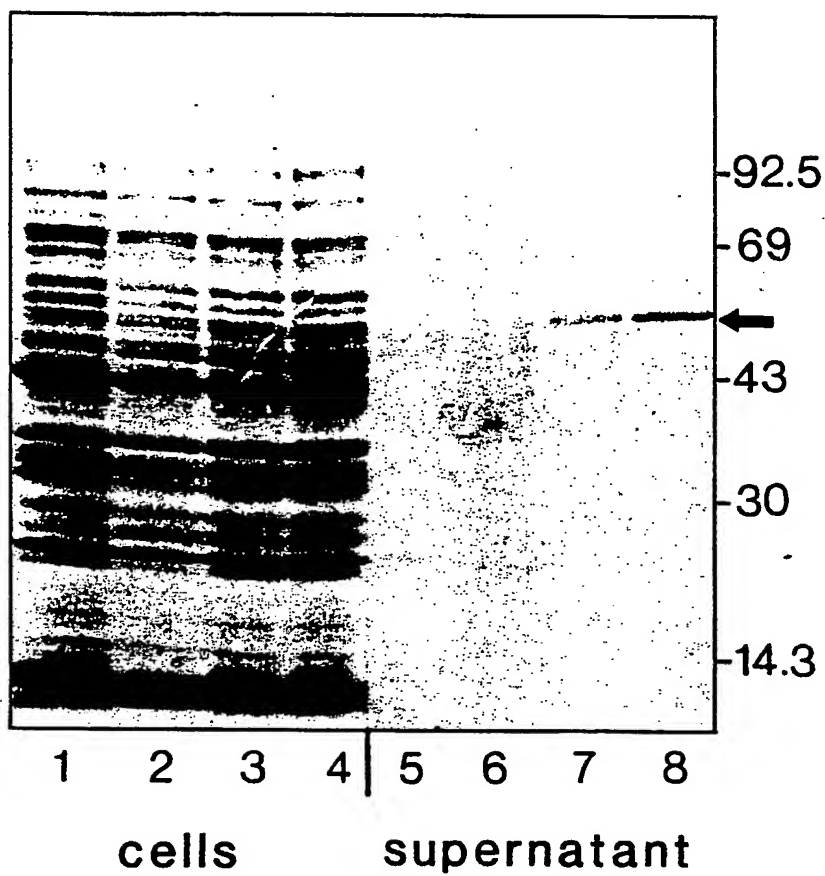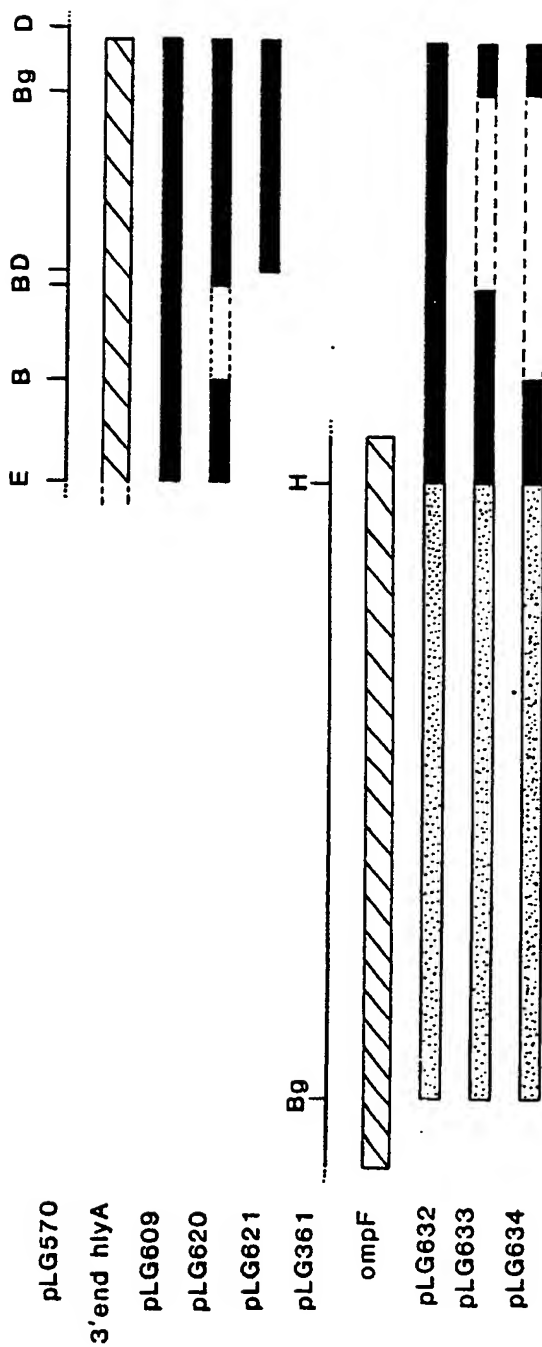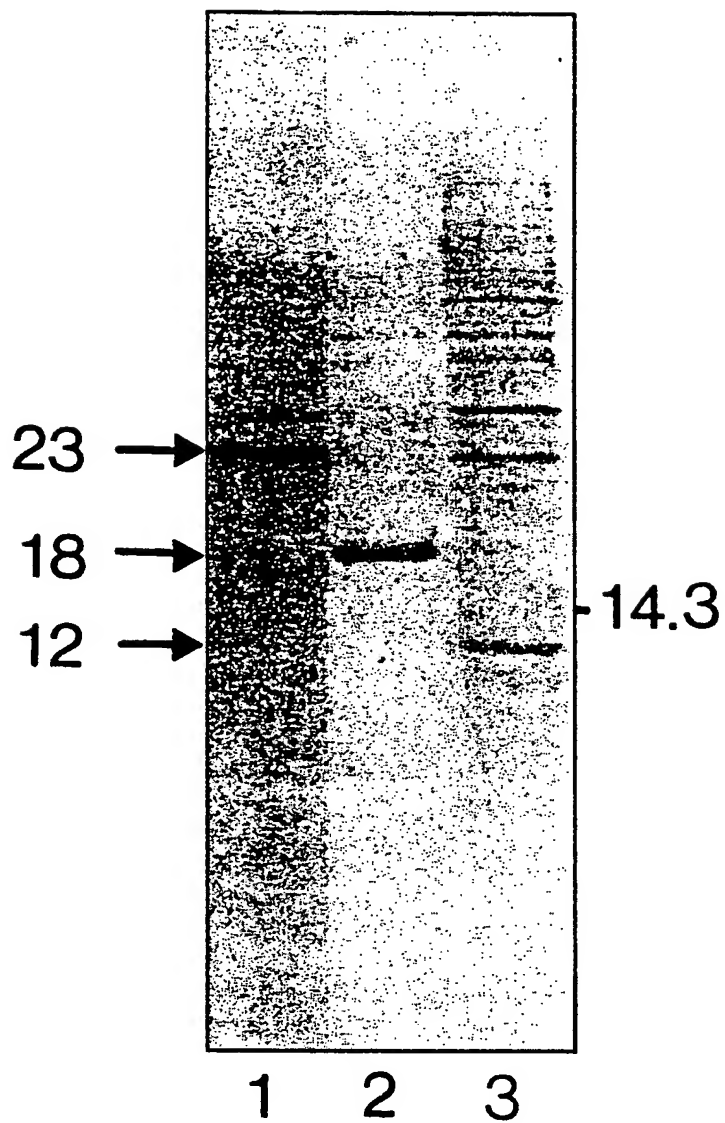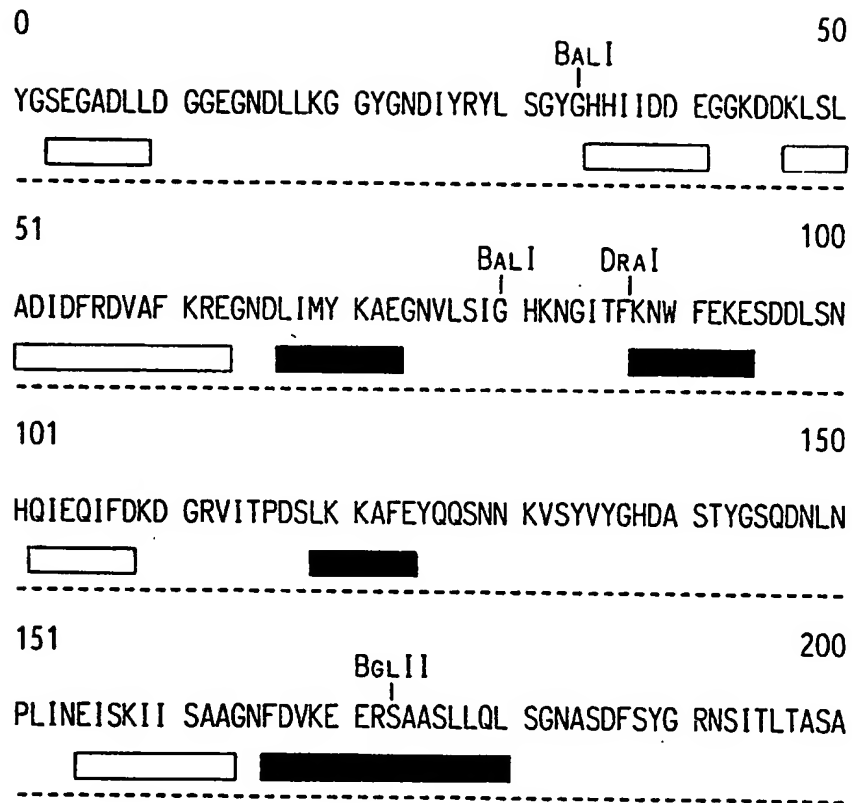
Fig.12
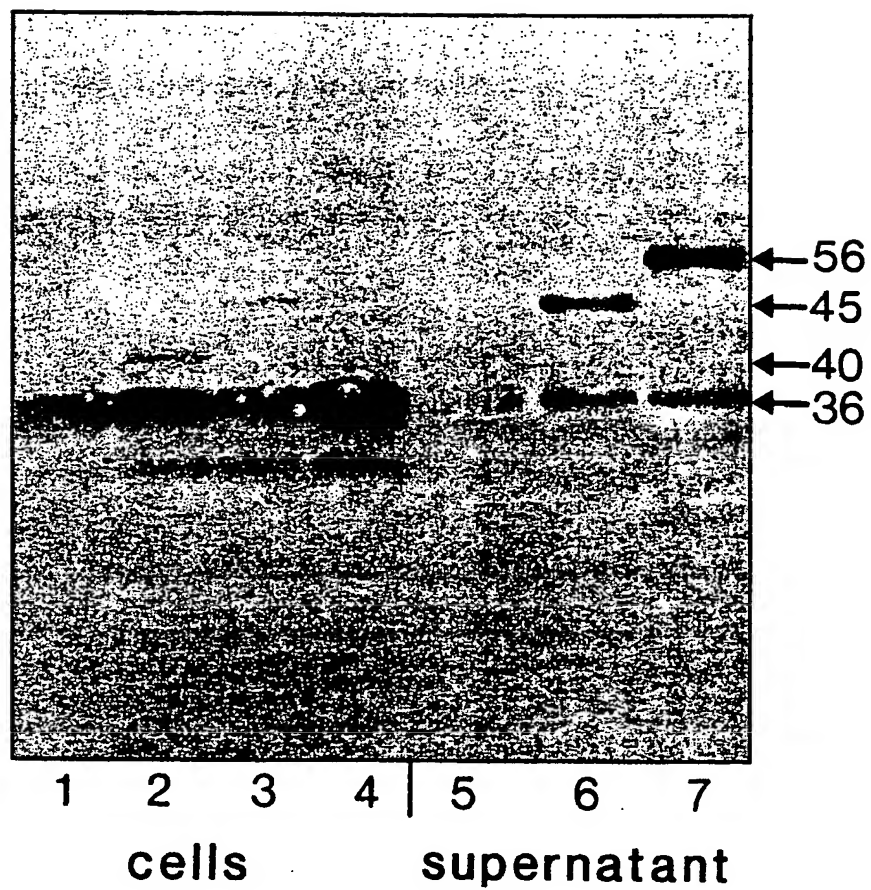
Fig.13

3 READING FRAMES OF E.COLI HAEMOLYSIN 23KD SECRETION SIGNAL
AVAILABLE FROM PROCHYMOSIN (PMG168) SMAI SITE

PPH - 1   CAG GAG CCC $\overset{\text{SMAI}}{|}$ GGG GAA AAT

PPH - 2   CAG GAG CCC $\overset{\text{SMAI}}{|}$ GG GAA AAT

PPH - 3   CAG GAG CCC $\overset{\text{SMAI}}{|}$ G GGA AAT

Fig.17

Fig.14

a) Fragment 1 EcoRI / Xma I
b) Fragment 2 EcoR I / Hind III

a) Fragment 3 (1.6 Kb)
Xma I / Hind III

3 Way Ligation

Fig.15

40 →   Supernatant

40 →   Cells

  1 2 3 4 5 | 1 2 3 4 5
  + Export | − Export
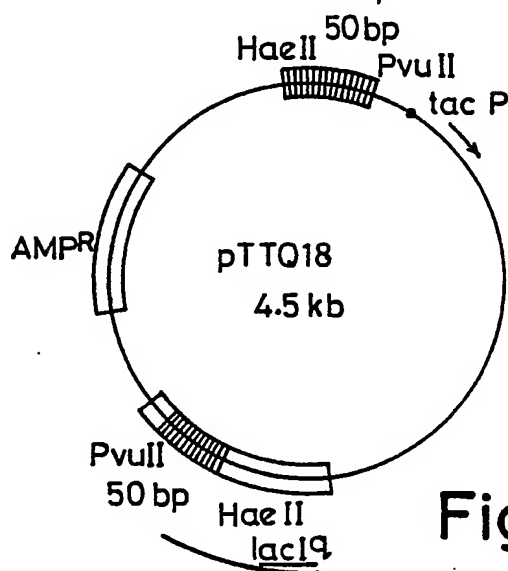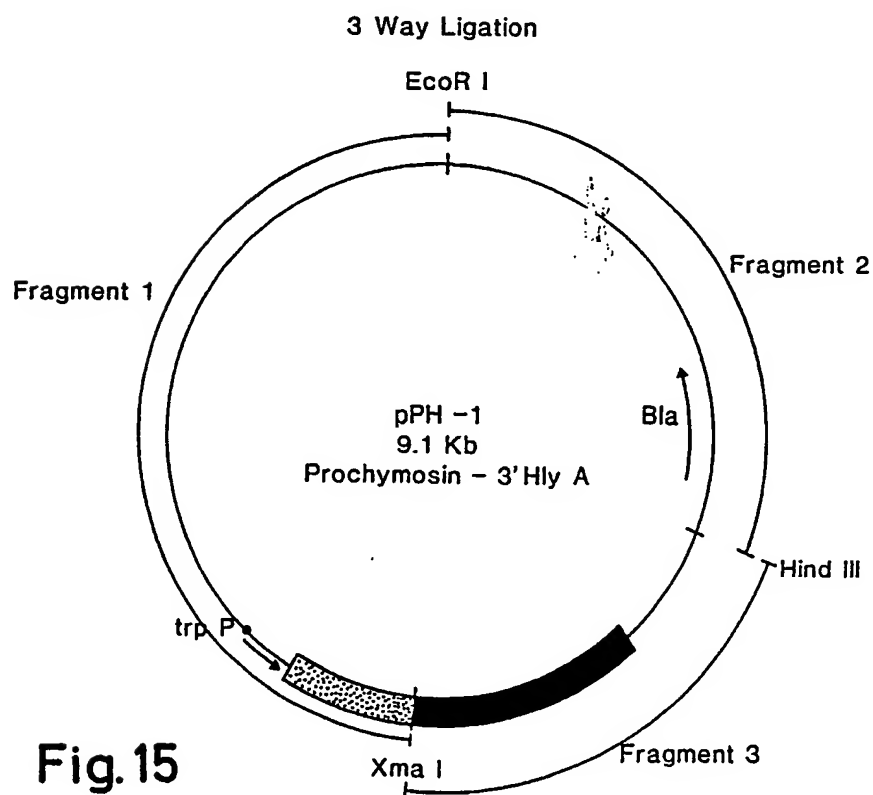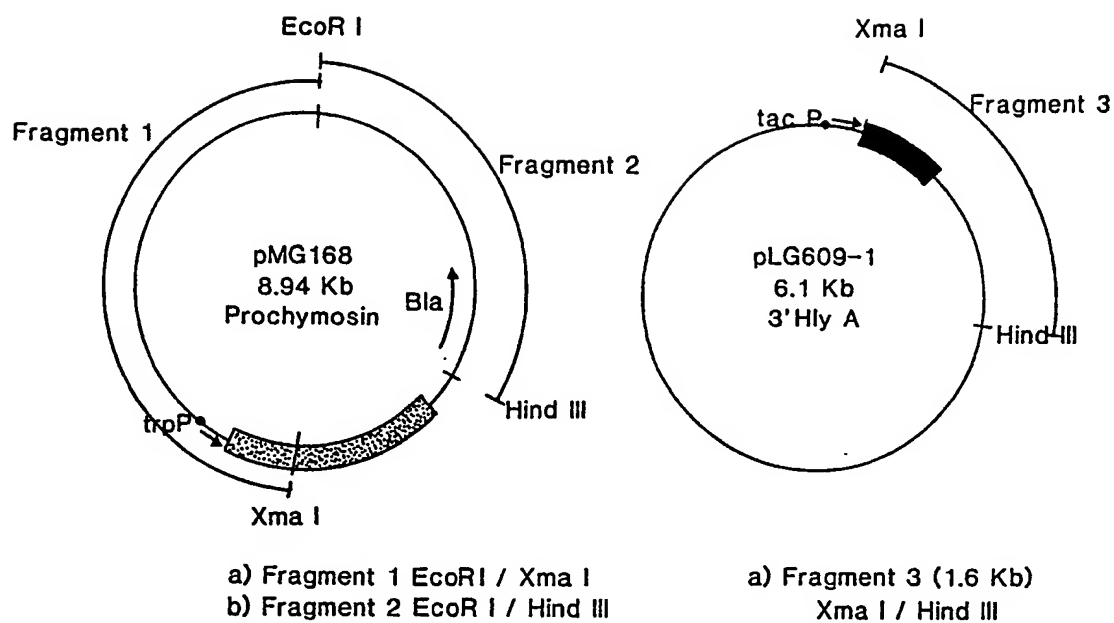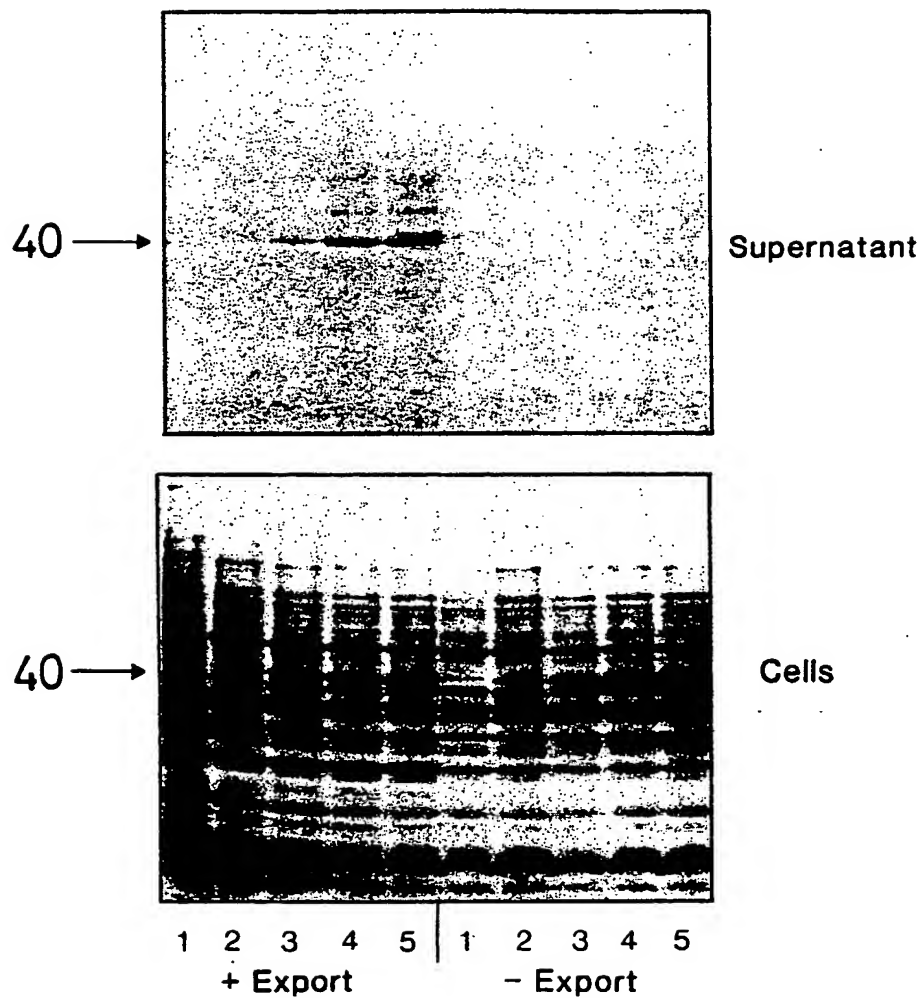
Fig.16

17/18
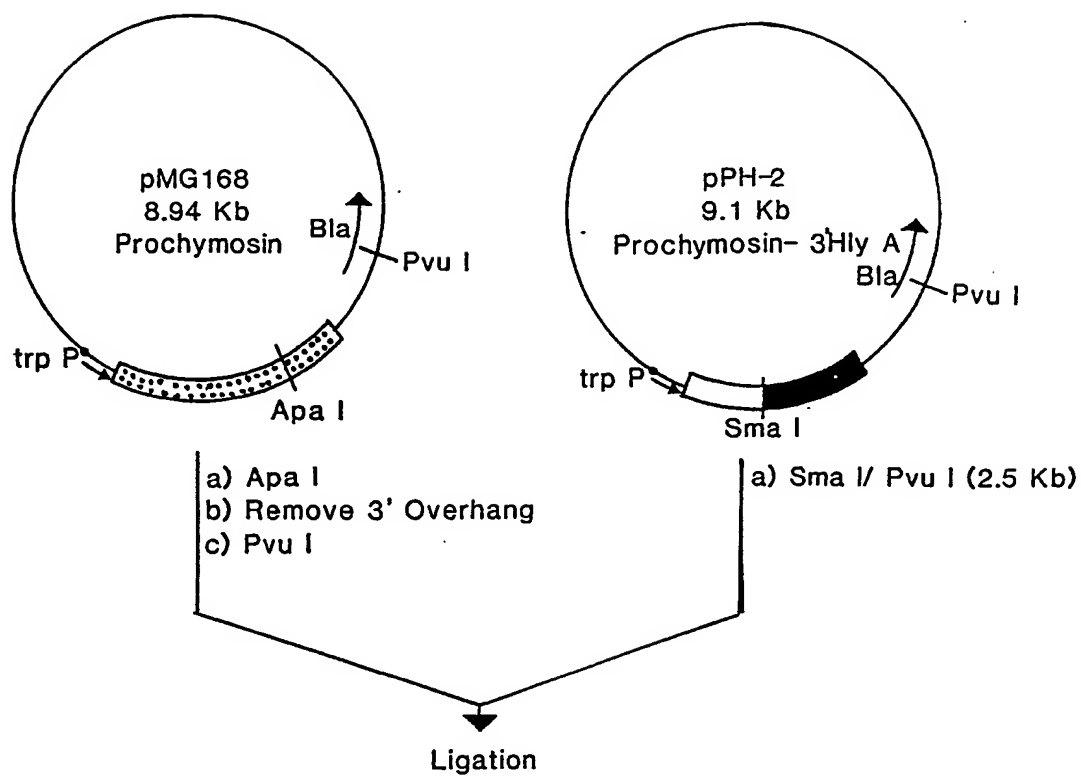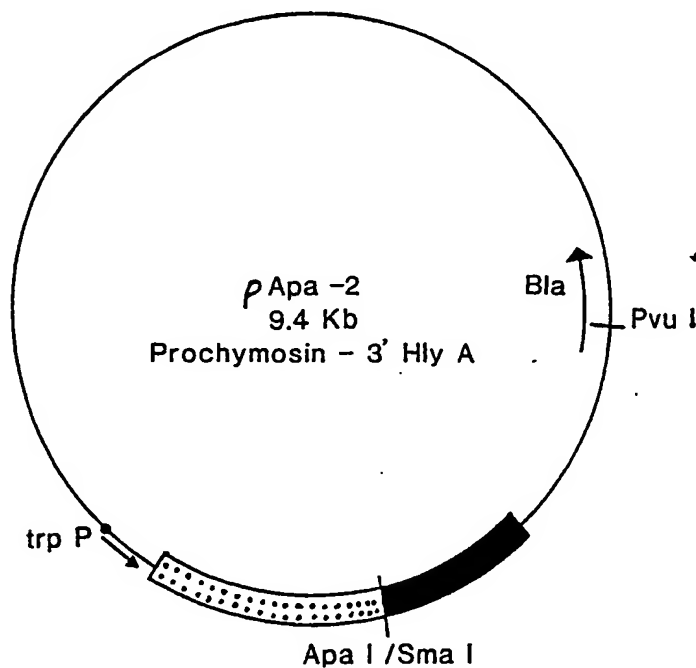
Fig.18
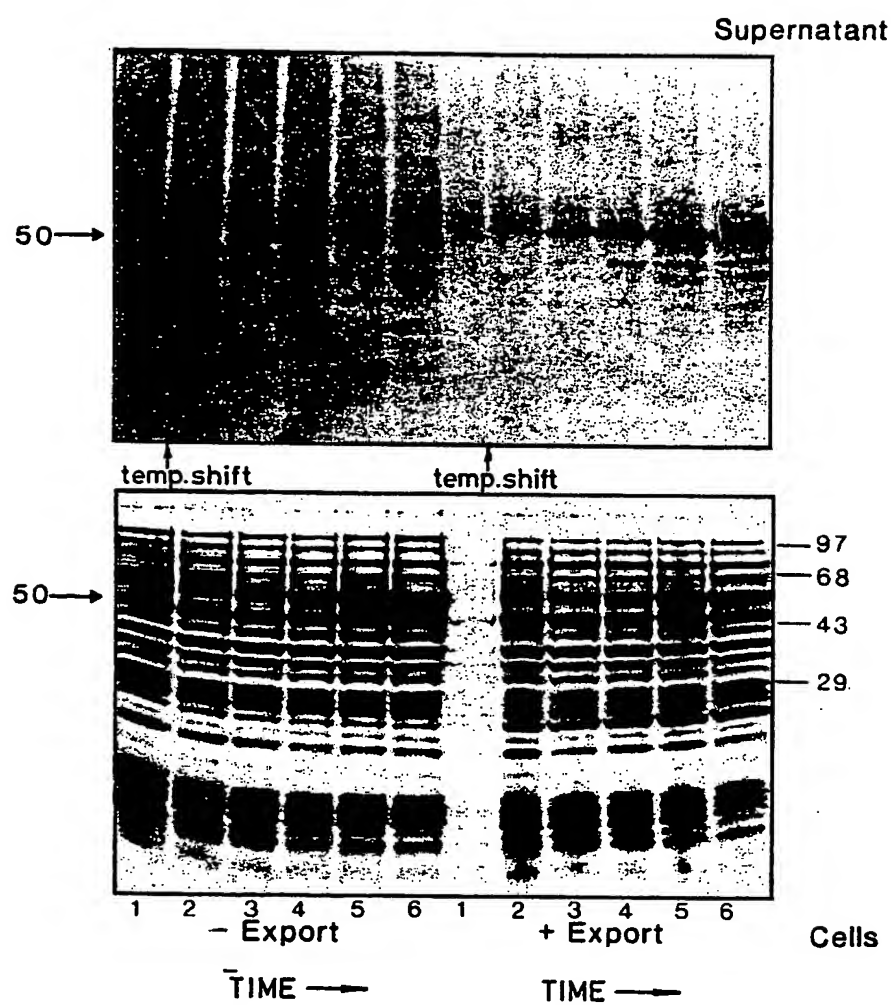
Fig.19

INTERNATIONAL SEARCH REPORT

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 N 15/00; C 12 P 21/02; C 12 N 1/20

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC⁴	C 12 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	Chemical Abstracts, vol. 101, no. 21, 19 November 1984 (Columbus, Ohio, US) N. Mackman et al.: "Functional characterization of a cloned hemolysin determinant from E.coli of human origin, encoding information for the secretion of a 107K polypeptide", see page 190, abstract no. 185197q, & MGG, Mol. Gen. Genet. 1984, 196(1), 129-34 (Eng) (cited in the application) --	
A	Chemical Abstracts, vol. 103, no. 13, 30 September 1985 (Columbus, Ohio, US) T. Felmlee et al.: "Nucleotide sequence of an Escherichia coli chromosomal hemolysin", see page 142, abstract no. 99555q, & J. Bacteriol. 1985, 163(1), 94-105 (Eng) (cited in the application) --	
A	Chemical Abstracts, vol. 103, no. 9, 2 September 1985 (Columbus, Ohio,	./.

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd July 1987	- 6 OCT 1987

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	L. ROSSI

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	US) T. Felmlee et al.: "Escherichia coli hemolysin is released extra-cellularly without cleavage of a signal peptide", see page 355, abstract. no. 67991n, & J. Bacteriol. 1985, 163(1), 88-93 (cited in the application) --	
P,A	Biotechnology, vol. 4, no. 5, May 1986 (New York, US) I. Barry Holland et al.: "Secretion of proteins from bacteria", pages 427-431, see page 429, column 2, line 27 - page 430, column 1, line 60 --	1-10
P,X	Mol. Gen. Genet., vol. 205, 1986 Springer-Verlag L. Gray et al.: "The carboxy-terminal region of haemolysin 2001 is required for secretion of the toxin from Escherichia coli", pages 127-133, see page 131, column 1, lines 29-43; page 132, column 1, line 8 - column 2, line 49 (cited in the application)	1-10